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A Green Approach: Optimization of the UPLC Method Using DoE Software for Concurrent Quantification of Pioglitazone and Dapagliflozin in a SNEDDS Formulation for the Treatment of Diabetes

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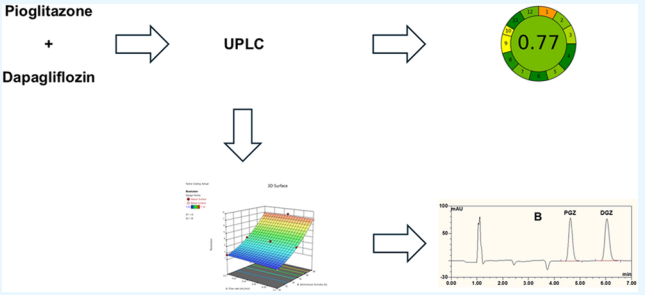
ABSTRACT: Clinical trials revealed that pioglitazone (PGZ) and dapagliflozin (DGZ) not only maintain normal blood glucose levels but also reduce complications of diabetes mellitus. To meet the demand for simultaneous measurement of these drugs in fixed combinations, an optimized and green UPLC method is required. The present study utilized Design of Experiments (DoE) software to optimize analytical parameters for simultaneous drug analysis. The method was validated for its linearity, accuracy, and precision. Furthermore, the drug content was estimated in different pharmaceutical dosage forms. Finally, Analytical Greenness (AGREE) software was utilized to assess the environmental sustainability of the optimized UPLC method. Drugs were successfully separated using optimized conditions on the C18 Acquity BEH column (2.1 mm × 100 mm, 1.7 μm) at a temperature of 45 °C. The mobile phase consisted of ethanol and 9 mM ammonium formate buffer (43.7:56.3), with elution carried out at a flow rate of 0.246 mL/min. The optimized method showed excellent linearity (R^2 > 0.999), accuracy (92.45−109.25%), and good precision (RSD < 6.27%) for both drugs. In addition, the optimized UPLC method was able to determine the drug content within the marketed pharmaceutical dosage form accurately. The developed UPLC method also prioritized eco-friendliness by using green solvents to minimize the negative impact on the environment. The green UPLC method provides a reliable and accurate approach to estimate PGZ and DGZ in a fixed diabetes treatment combination. It promotes sustainable lab practices and paves the way for analytical methods for new dose combinations.



1. INTRODUCTION

According to the World Health Organization, diabetes is a chronic metabolic disorder characterized by an elevation in the level of blood glucose. This could result in significant injury to the heart, kidneys, nerves, blood vessels, and eyes.[1] Approximately 422 million individuals worldwide are affected by diabetes, with Type 2 diabetes being the most prevalent form.[2] This form of diabetes typically arises due to a combination of insulin deficiency and resistance.[3] Managing diabetes involves several measures, including controlling the diet and exercising. However, administration of medication may be necessary to control normal blood glucose levels and prevent diabetes-related complications.[4]

Thiazolidinediones (TZDs) are antihyperglycemic drugs used for regulating glucose levels in Type 2 diabetes.[5] Pioglitazone (PGZ), a TZD, has shown efficacy in improving pancreatic β-cell function and reducing glycosylated hemoglobin levels.[6,7] It also reduces the risk of hypoglycemia and cardiovascular events in high-risk patients.[8,9] However, weight gain is a common drawback of PGZ.[10] To address this issue, combining sodium-glucose cotransporter-2 inhibitors (SGLT2-i) with PGZ has effectively achieved antihyperglycemic effects while mitigating the weight gain associated with PGZ.[11,12] This combination has additional benefits, such as reducing heart failure risk,[13] visceral fat and body weight,[14] and dyslipidemia.[15] Furthermore, the combination is more effective in preserving renal function and preventing nephropathy progression.[16]

Administering a fixed combination dose overcomes challenges associated with multiple tablets, such as delayed or missed doses, adherence issues, and reduced treatment effectiveness.[17,18] However, according to an extensive literature survey, there is currently no available UPLC method for the

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simultaneous determination of PGZ and dapagliflozin (DGZ) using the green method. This research gap highlights the need to develop a method for concurrently determining PGZ and DGZ in the proposed combination.

Managing multiple parameters in the past has been time-consuming, especially when chromatographic conditions are adjusted to achieve desired peak parameters. Therefore, an undefined number of runs could be required to optimize the UPLC method.[19] In this regard, the Design of Experiments (DoE) could be utilized to follow the principle of green chemistry fully. This is achieved through understanding the impact of multiple parameters on the optimized condition for the UPLC method.[20] Implementing DoE minimizes analysis trials, leading to cost savings, reduced environmental hazards, and more efficient routine analysis.[21]

Green analytical chemistry (GAC) has gained popularity in modern analytical chemistry as it reduces the usage of toxic solvents and energy consumption.[22,23] Therefore, in the present study, ethanol was selected as a safe solvent instead of toxic solvents such as acetonitrile and methanol to develop an eco-friendly liquid chromatographic method.[24,25] Various evaluation methods have been employed to assess the environmental sustainability of different analytical results to achieve these objectives. The methodologies used in this study include the Analytical Eco-Scale (AES) approach, the National Environmental Methods Index (NEMI), the Red-Green-Blue (RGB) approach, the Analytical Greenness (AGREE) metric approach, and the Green Analytical Procedures Index (GAPI).[22] The AES, GAPI, NEMI, and RGB approaches were observed to rely on specific values of the Green Analytical Criteria (GAC).

The present study aims to develop an eco-friendly method for the simultaneous determination of PGZ and DGZ as a proposed combination for the treatment of diabetes. Optimized conditions were selected based on the minimum retention time, maximum peak area, tailing factor close to 1, plate number above 2000, and resolution of two peaks with a value of more than 2. The optimized UPLC method was validated in terms of linearity, accuracy, and precision. Finally, the environmental sustainability of the developed method was evaluated by using AGREE software.

2. MATERIALS AND METHODS

2.1. Materials. Riyadh Pharma, located in Riyadh, KSA, generously provided pioglitazone (PGZ) and dapagliflozin (DGZ) used in this study. The certificates of analysis (COA) showed that PGZ and DGZ purity was 100.1 and 99.9%, respectively, and was in compliance with relevant specifications. HPLC-grade ethanol was acquired from Fisher Scientific, Bishop Meadow Road, U.K. Ammonium formate was acquired from Sigma-Aldrich. All other reagents and chemicals were of analytical grade.

2.2. UPLC Method. *2.2.1. UPLC Instrumentation.* The Dionex UPLC system (Thermo Scientific, Bedford, MA) was utilized to develop and validate the analytical method. The mobile phase was eluted by using the Dionex pump system through a column placed in the Dionex column oven chamber. All samples were injected using the Dionex automatic sample manager through a connected column. The spectrum of drugs in samples was obtained by using a Dionex photodiode array (PDA) detector, and peak analysis was performed by using the Chromeleon Client program.

2.2.2. Initial Screening Using the One-Factor-at-a-Time (OFAT) Approach. Preliminary initial screening experiments were conducted to identify the independent variables for the present study. The parameters that were systematically evaluated during the OFAT screening included the column selection, buffer type and concentration, mobile phase composition, and column temperature. For each parameter, the following criteria were used to assess the chromatographic performance:

- Peak resolution: minimum resolution of 2.0 between adjacent peaks
- Peak symmetry: tailing factor between 0.9 and 1.2
- Retention time: aiming for efficient separation within a reasonable run time (<10 min)
- Peak area: maximizing the peak area for improved sensitivity

Various columns were tested for drug separation, including the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm), the Acquity Waters Hilic column (50 mm × 2.1 mm, 1.7 μm), and the Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm). The column temperature was maintained at 45 °C to mitigate the pressure generated by ethanol and to safeguard the column from high pressure. Additionally, different buffers, such as 0.1% formic acid and 10 mM ammonium formate, were evaluated at various ratios to investigate the effect of pH on drug separation. In addition, acetonitrile was mixed with the aqueous phase (a mixture of 0.1% formic acid and 10 mM ammonium formate) using the Dionex pump system to establish the optimal design space. The flow rate of the mobile phase was varied between 0.2 and 0.4 mL/min to determine the best conditions for the analysis.

2.2.3. Chromatographic Condition. Reverse-phase isocratic elution mode was utilized by using a mobile phase composed of a mixture of ethanol and a 9 mM ammonium formate solution (pH 3.7) in various ratios. The mobile phase was eluted at a constant rate through the connected Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at the assigned temperature. The injection volume was 2 μL, and the drug absorbance for both drugs was measured at 223 nm.

2.2.4. Preparation of the Stock Solution, Standard Solutions, and Quality Control Samples (QCs). A standard stock solution of PGZ and DGZ, with a concentration of 500 μg/mL, was prepared by dissolving 5 mg each of PGZ and DGZ in a 10 mL volumetric flask using methanol. During the optimization process, standard working solutions containing drugs at a concentration of 50 μg/mL were prepared from a standard stock solution following appropriate dilution. For the validation process, various concentrations ranging from 50.0 to 1.0 μg/mL were prepared following dilution of the standard stock solution. Quality control samples, consisting of a low limit of detection and low, intermediate, and high concentrations, were also prepared following the same procedure.

2.3. Method Optimization Using DoE. *2.3.1. Effect of Independent Parameters.* Design of Experiments (DoE) software (Version 13), particularly response surface methodology using a central composite design, was used to study the impact of numerous independent analytical factors on the measured responses. The influence of two independent factors was studied, including the flow rate and ratio of ammonium formate buffer in the ranges of 0.2−0.3 mL/min and 50−60%, respectively (Table 1). Those independent factors and their ranges were carefully selected for DoE based on our

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Table 1. Suggested Analytical Procedure by Design of Experiments Software

	independent factors	
run	flow rate (mL/min)	ammonium formate buffer (%)
1	0.25	55
2	0.25	55
3	0.3	60
4	0.2	60
5	0.2	55
6	0.3	50
7	0.25	55
8	0.3	55
9	0.25	55
10	0.25	50
11	0.25	60
12	0.2	50
13	0.25	55

preliminary experimental assessment and prior knowledge. Various responses, including the retention time, peak area, tailing factor, and plate number for both drugs and the resolution of the two drug peaks from each other, were measured. The suggested 13 experiments were run using the Dionex UPLC system. The impact of independent factors on the measured responses was assessed via the analysis of variance (ANOVA). Analysis of the data was conducted using Design Expert software (version 13).

2.3.2. Method Optimization. The optimized analytical conditions for the simultaneous analysis of PGZ and DGZ were determined based on specific criteria for the chromatographic parameters. These criteria included the minimum retention time, maximum peak area, tailing factor close to 1, plate number above 2000, and resolution of two peaks with a value of more than 2. This ensures quick elution and separation of the two drug peaks with symmetrical peak shapes for the accurate quantification of PGZ and DGZ.

2.3.3. Design Validation. The validation of the recommended optimized conditions involved comparing the predicted values obtained from the software to the actual values of the parameters. The reliability and accuracy of the design can be assessed by evaluating the extent to which the actual values fall within 95% prediction intervals of the predicted response value. A close agreement between the predicted and actual values within the prediction interval range indicates the robustness and reliability of the design's ability to estimate the response accurately.

2.4. Validation of the Optimized UPLC Method. The optimized analytical method was subjected to an assessment of its suitability through testing of various validation parameters. These characteristics, which encompassed specificity, accuracy, precision (% CV), linearity, detection limit (DL), and quantitation Limit (QL), were evaluated in compliance with the recommended guidelines outlined by the International Conference of Harmonization (ICH Q2 R1) for validation of analytical procedures.[26]

The constructed calibration curve was used to estimate the linearity of the calibration curve. Based on the average of six runs, the theoretical concentration was plotted against the measured peak area of the injected standard solution. The

Table 2. Acceptance Criteria for Measured Parameters

parameter	AOAC standards	
linearity	The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample.	
	The correlation coefficient (r) should typically be ≥0.99 across the concentration range.	
specificity/selectivity	It is the probability of the method giving a (−) response when the sample is truly without the analyte; it must be confirmed through tests like blank sample analysis or spiking experiments.	
range	A sample that contains the analyte at some positive concentration.	
	Range of concentration where $c > 0$	
accuracy	It is defined as the ratio of the observed mean test result to the true value; the range of the acceptable mean recovery expands as the concentration of the analyte decreases.	
	Total % recovery = $100(C_f)/(C_u + C_A)$	
	where C_f = concentration of fortified samples, C_u = concentration of unfortified samples, and C_A = concentration of the analyte added to the test sample.	
	analyte concentration	**mean recovery %**[a]
	1 ppm	80−110
	10 ppm	80−110
	100 ppm	90−107
	1000 ppm	95−105
repeatability or intra-assay precision	The precision of a method is the closeness of agreement between independent test results obtained under stipulated conditions; precision is usually expressed in terms of imprecision and computed as a relative standard deviation of the test results; the imprecision of a method increases as the concentration of the analyte decreases.	
	analyte concentration	**RSD %**[a]
	1 ppm	11
	10 ppm	7.3
	100 ppm	5.3
	1000 ppm	3.7

[a]Table excerpted from AOAC Peer-Verified Methods Program, Manual on Policies and Procedures (1998), AOAC International, Rockville, MD.

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regression coefficient (R^2) was calculated by using Excel software.

The DL and QL were estimated based on the determination of the slope of the calibration curve and the standard deviation (σ) of responses[27] using the following equations

$$\mathrm{DL} = 3{\cdot}3\frac{\sigma}{\mathrm{slope}} \quad (1)$$

$$\mathrm{QL} = 10\frac{\sigma}{\mathrm{slope}} \quad (2)$$

The slope was estimated from the calibration curve of the analyte. The estimate of σ was determined based on the calibration curve where the standard deviation of y-intercepts of regression lines was used as the standard deviation.

The accuracy of the developed optimized analytical method was calculated to define how close the mean results were to the theoretical concentration of the drug in the samples. In addition, the precision was calculated to determine how close the estimated drug concentrations for the same sample were to each other. Accuracy (% recovery) and precision (% RSD) were calculated for quality control samples: QL, low level, medium level, and high level for each drug. The method validation was performed in accordance with the Association of Official Analytical Chemists (AOAC International) guidelines[28] to ensure that our method adheres to recognized standards in food and drug analysis. The acceptance criteria adopted in this work are given in Table 2.

2.4.1. Test of Homoscedasticity. The homoscedasticity test is an important component of method validation in analytical chemistry, particularly when assessing linearity. Homoscedasticity, also known as homogeneity of variance, is the assumption that the variability of a variable is uniform across the range of values of the second variable that predicts it. In the context of our UPLC method validation, this ensures that the variability of the response (peak area) is consistent across the entire range of analyte concentrations. It also validates the assumption that the error terms (residuals) have a constant variance, which is crucial for the proper application of linear regression. Moreover, it helps to confirm the reliability and accuracy of the method across the entire concentration range, enhancing the overall robustness of the validation process.[29]

2.5. AGREE for Assessment of Method Greenness. The "AGREE" methodology was employed to measure the level of environmental sustainability by evaluating twelve greenness assessment criteria (GAC) and assigning the corresponding scores. The investigation of the UPLC method showcased its environmental friendliness through the implementation of a lowered flow rate (0.246 mL/min) and green solvents (ethanol and water). The observed relationship in this system demonstrated linearity over the concentration ranges from 2.5 to 50 μg/mL and from 3.5 to 50 μg/mL for PGZ and DGZ, respectively.

2.6. Application of the Developed UPLC Method. *2.6.1. Application on Marketed Tablets.* PGZ and DGZ contents within tablet dosage forms (Glados and Forxiga, respectively) were estimated using the developed UPLC method. Each tablet was crushed to obtain a uniform powder and ensured complete drug extraction. The crushed tablet was placed in a 50 mL volumetric flask and diluted with methanol to a volume. Following 5 min of sonication, the mixture was subjected to centrifugation for 5 min at 10,000 rpm to precipitate the undissolved particles. The drug concentration in the supernatant was determined following an appropriate dilution.

2.6.2. Application on the Prepared Self-Nanoemulsifying Drug Delivery System (SNEDDS) Formulation. A self-nanoemulsifying drug delivery system (SNEDDS) formulation was prepared to be loaded with pioglitazone (PGZ) and dapagliflozin (DGZ). First, Tween-80, propylene glycol, and Imwitor-308 were weighed and mixed using a magnetic stirrer in a ratio of 5:3.5:1.5 to prepare a drug-loaded SNEDDS formulation. The formulation was then loaded with the drugs at concentrations of 30 mg/g for PGZ and 20 mg/g for DGZ, respectively. Drug concentrations within the SNEDDS formulation were estimated using the developed UPLC method to determine the accuracy of the developed method. For further investigation, the formulation was dispersed in distilled water to visualize the physical appearance of the SNEDDS.

2.6.3. Effect of the Matrix on Accuracy and Precision. To evaluate the impact of the formulation matrix (SNEDDS) on the accuracy and precision of the drug measurements, we conducted a back calculation study. Standard solutions of PGZ and DGZ at concentrations of QL, as well as low and high levels of drug concentrations, were prepared with the inclusion of the SNEDDS. The drug concentrations in the prepared samples were then estimated against a calibration curve. The accuracy and precision of these measurements were assessed to ensure that the presence of the SNEDDS matrix had a negligible effect. This method aims to prove that the SNEDDS matrix components do not significantly influence the accuracy and precision of the drug concentration measurements.

3. RESULTS AND DISCUSSION

3.1. Initial Screening Results of the One-Factor-at-a-Time (OFAT) Approach. The initial screening was conducted using the one-factor-at-a-time (OFAT) approach. This method was chosen for its simplicity and ability to provide a clear understanding of how individual factors affect the chromatographic separation. The primary goal was to determine suitable ranges for further optimization using Design of Experiments (DoE) studies rather than finalizing the method at this stage. Table 3 shows the detailed scheme that has been followed to identify the independent variables for the subsequent Design of Experiments (DoE) optimization process. Our screening results revealed that the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) provided optimal separation of both drugs, exhibiting symmetrical peak shapes. This led to its selection as the stationary phase for further method development. In terms of the mobile phase composition, a 9 mM ammonium formate solution (pH 3.7) was found to be most effective in separating the target compounds on the chosen column, and it was thus selected as the aqueous component of the mobile phase. Further exploration of the mobile phase composition, specifically the ratio between ethanol and the ammonium formate solution, showed that reducing the proportion of the ammonium formate solution below 50% resulted in an inadequate peak resolution, while increasing it above 60% led to undesirably extended run times. Consequently, we identified a 50−60% range of the ammonium formate solution as optimal for further investigation. The column temperature was set at 45 °C, a decision made to mitigate the pressure generated by ethanol while also protecting the column from excessive stress. Based on these initial findings, we identified the ratio of aqueous buffer and

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Table 3. Parameters Evaluated for Initial Screening

	column selection	buffer type and concentration	mobile phase composition	column temperature
scope of testing	• Acquity UPLC BEH C18 column • Acquity Waters Hilic column • Acquity UPLC HSS T3 column	0.1% formic acid, 10 mM ammonium formate, various pH levels.	various ratios of ethanol and ammonium formate solution	25−45 °C
results	the Acquity UPLC BEH C18 column provided the best separation of both drugs with symmetrical peak shapes.	9 mM ammonium formate solution (pH 3.7) effectively separated both drugs on the selected column.	• ammonium formate < 50%: poor peak resolution • ammonium formate > 60%: prolonged run times	45 °C provided optimal separation while minimizing the pressure generated by ethanol
decision	the selected Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) as the stationary phase.	chose 9 mM ammonium formate solution (pH 3.7) as the aqueous phase.	selected 50−60% ammonium formate solution as the optimal range for further optimization	set the column temperature to 45 °C

the flow rate as the key independent variables for the subsequent optimization process using DoE. The DoE approach was then employed to fine-tune these parameters within the ranges established during the initial screening, allowing for a more comprehensive exploration of their interactive effects on the chromatographic performance.

3.2. Studying the Influence of Independent Factors. DoE was utilized to study the impact of the independent factors (the ratio of aqueous buffer and the flow rate) on the various analytical response parameters including the retention time, peak area, tailing factor, and plate number for both drugs (PGZ and DGZ), as well as the resolution of two peaks. The suggested thirteen experimental runs, including five center points, by DoE were achieved using the UPLC instrument. Table 4 shows the results of the measured response values obtained from the experimental runs.

The impact of independent factors on the measured analytical parameter was determined individually based on numerous mathematical models. Following ANOVA analysis, the selected model by DoE software is presented in Table 5. The software selection is based on the model having a high predicted and adjusted R^2 value with a difference less than 0.2, a high p-value, and with a nonsignificant lack of fit. The significance of the selected model for each response indicated by the calculated p-value is less than 0.0001.[30] The quartic model was suggested for the retention time of PGZ and DGZ, the peak area of PGZ, the plate number of PGZ and DGZ, and the resolution of two drug peaks, while the linear model was suggested for the peak area of DGZ and the tailing factors of PGZ and DGZ.

3.2.1. Retention Time. The detected retention times of PGZ and DGZ ranged from 2.64 to 7.40 min and from 2.91 to 10.77 min, respectively (Table 4). ANOVA analysis revealed that the flow rate and percent of ammonium formate buffer in the mobile phase had a significant impact on the retention time of both drugs (Table 6). The impact of two independent factors on the measured retention time is shown in Figure 1A,B, while the magnitude of their impact can be estimated from the value of the factor's coefficient in polynomial equations (eqs 3 and 4). Overall, increasing the flow rate and decreasing the percent of ammonium formate buffer in the mobile phase resulted in a significant reduction in the measured retention time with a pronounced impact of the latter.

$$\begin{aligned}&\text{retention time (PGZ)}\\&= 4.22 - 0.81\ \text{flow rate} + 1.53\%\ \text{ammonium formate}\\&\quad - 0.24 \times \text{flow rate} \times \%\ \text{ammonium formate} + 0.16\\&\quad \text{flow rate}^2 + 0.38\%\ \text{ammonium formate}^2\end{aligned} \tag{3}$$

$$\begin{aligned}&\text{retention time (DGZ)}\\&= 5.22 - 1.13\ \text{flow rate} + 2.71\%\ \text{ammonium formate}\\&\quad - 0.49 \times \text{flow rate} \times \%\ \text{ammonium formate} + 0.22\\&\quad \text{flow rate}^2 + 0.92\%\ \text{ammonium formate}^2\end{aligned} \tag{4}$$

The observed reduction in the retention time with the increasing flow rate is attributed to an increase in velocity of the mobile phase. This resulted in increasing elution of drugs from the column and a reduced retention time.[31] On the contrary, increasing the ammonium formate ratio in the mobile phase resulted in a significant increment in the retention time. This could be attributed to the retention of drugs on the

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Table 4. Measured Analytical Responses for the Suggested Runs[a]

	responses								
	RT		PA		TF		PN		
run	(PGZ)	(DGZ)	(PGZ)	(DGZ)	(PGZ)	(DGZ)	(PGZ)	(DGZ)	resolution
1	4.26	5.18	5.17	6.06	1.09	1.14	3632	4351	3.08
2	4.28	5.21	4.93	6.24	1.11	1.13	3726	4468	3.13
3	5.28	7.43	4.43	5.05	1.05	1.09	5494	6973	6.72
4	7.40	10.77	6.23	7.17	0.95	0.97	4559	6010	6.81
5	5.15	6.46	6.84	7.36	1.08	1.10	3402	4421	3.54
6	2.64	2.91	4.06	4.62	1.24	1.36	2879	2993	1.35
7	4.20	5.24	5.11	6.21	1.13	1.17	3770	4366	3.53
8	3.58	4.41	4.34	4.99	1.20	1.21	3964	4803	3.44
9	4.24	5.27	5.14	6.22	1.15	1.17	3872	4743	3.55
10	3.12	3.50	5.13	5.74	1.22	1.25	2700	2983	1.55
11	6.05	8.77	5.15	6.93	1.00	1.05	5311	6701	7.16
12	3.79	4.31	6.49	7.45	1.16	1.22	2700	3012	1.71
13	4.16	5.22	5.08	6.17	1.12	1.16	3790	4451	3.63

[a]RT: retention time, PA: peak area, TF: tailing factor, and PN: plate number.

Table 5. ANOVA Analysis of the Measured Responses for the Selected Models

response		selected model	freedom degree	adjusted R^2	predicted R^2	F value	p-value
retention time	PGZ	quadratic	5	0.9977	0.9914	1062.9	<0.0001
	DGZ	quadratic	5	0.9984	0.9909	1462.6	<0.0001
peak area	PGZ	quadratic	5	0.9655	0.8653	68.1	<0.0001
	DGZ	linear	2	0.9204	0.8505	70.4	<0.0001
tailing factor	PGZ	linear	2	0.9306	0.8973	81.4	<0.0001
	DGZ	linear	2	0.9689	0.9552	187.9	<0.0001
plate number	PGZ	quadratic	5	0.9855	0.9433	163.8	<0.0001
	DGZ	quadratic	5	0.9866	0.9699	177.1	<0.0001
resolution		quadratic	5	0.9855	0.9697	164.0	<0.0001

Table 6. p-Value Obtained from ANOVA Analysis That Presents the Impact of Two Independent Factors on the Measured Responses

measured response		p-value of the flow rate	p-value of % ammonium formate
retention time	PGZ	<0.0001	<0.0001
	DGZ	<0.0001	<0.0001
peak area	PGZ	<0.0001	0.7276
	DGZ	<0.0001	0.0576
tailing factor	PGZ	0.0002	<0.0001
	DGZ	<0.0001	<0.0001
plate number	PGZ	0.0003	<0.0001
	DGZ	0.0085	<0.0001
resolution of two peaks		0.3709	<0.0001

hydrophobic column owing to reduction in the organic phase ratio and the hydrophobic nature of drugs.[32,33]

3.2.2. Peak Area. The peak areas of PGZ and DGZ ranged from 4.06 to 6.23 mAU*min and 4.62 to 7.45 mAU*min, respectively (Table 4). In addition, the ANOVA analysis presented in Table 6 revealed that the ratio of ammonium formate buffer in the mobile phase did not significantly influence the measured peak areas of both drugs. However, it showed that the peak area of both drugs was significantly affected by the flow rate of the mobile phase. Furthermore, Figure 2A,B show the independent factors' impact on the peak area, and eqs 5 and 6 determine the magnitude of the factors' impact on the measured response. It was found that the peak area of drugs increased with the decreasing flow rate of the mobile phase, indicated by the negative sign. However, a positive sign of the ammonium formate percent indicates its agonistic effect on the peak area of the drugs with no significant influence.

$$\text{peak area (PGZ)} = 5.13 - 1.12\ \text{flow rate} + 0.02\%\ \text{ammonium formate} + 0.16 \times \text{flow rate} \times \%\ \text{ammonium formate} + 0.34\ \text{flow rate}^2 - 0.11\%\ \text{ammonium formate}^2 \quad (5)$$

$$\text{peak area (DGZ)} = 6.17 - 1.22\ \text{flow rate} + 0.22\%\ \text{ammonium formate} \quad (6)$$

Peak area is an essential parameter during the optimization of the analytical method owing to its impact on the sensitivity method.[34] The observed increment in the peak area while decreasing the flow rate could be attributed to the better interaction between the drugs and the column, which resulted in an enhanced peak resolution with a higher signal detection.[35] The obtained results are in agreement with previously published data by Mandpe et al.[36] The study revealed that reducing the flow rate of the mobile phase had a significant positive impact on the measured peak area of the drug. However, altering the ratio of the mobile phase compositions did not show any significant effect on the peak area.

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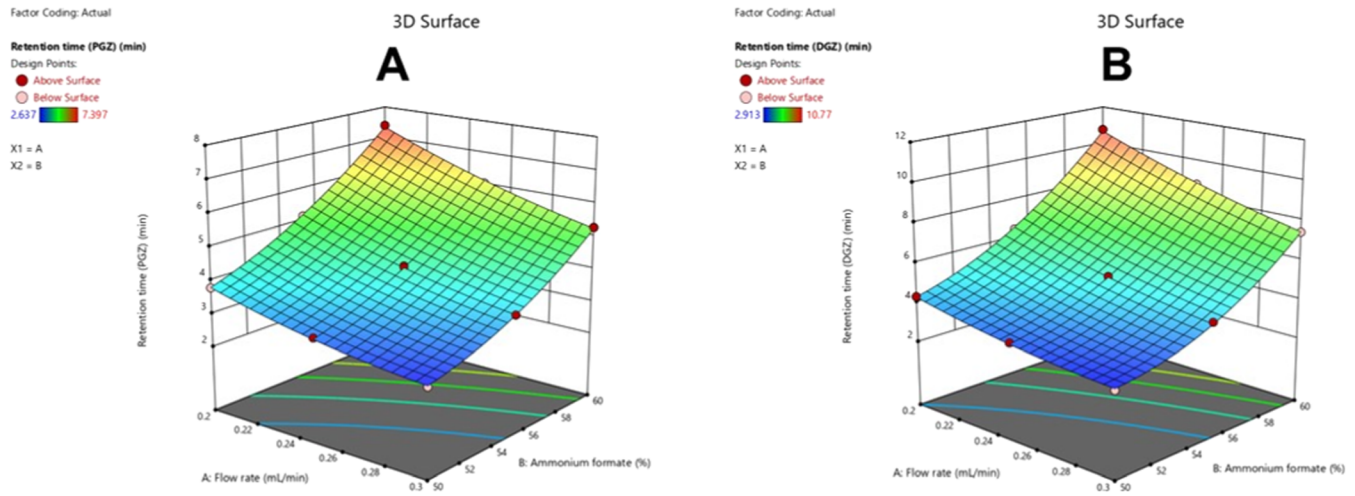

Figure 1. Impact of the flow rate and percent of ammonium formate buffer in the mobile phase on the retention times of (A) PGZ and (B) DGZ.

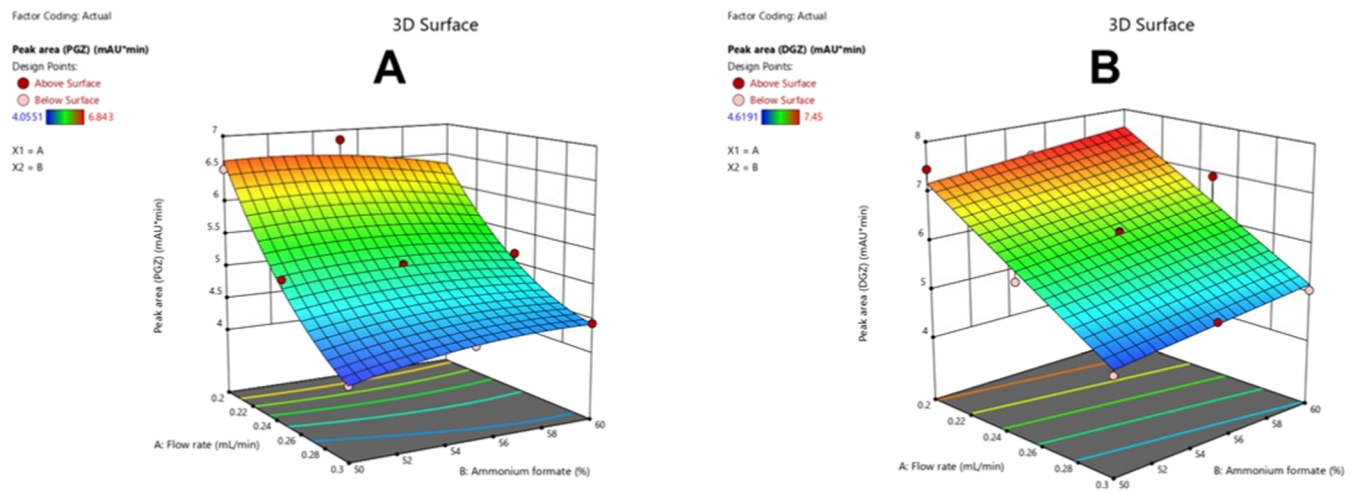

Figure 2. Impact of the flow rate and percent of ammonium formate buffer in the mobile phase on the peak areas of (A) PGZ and (B) DGZ.

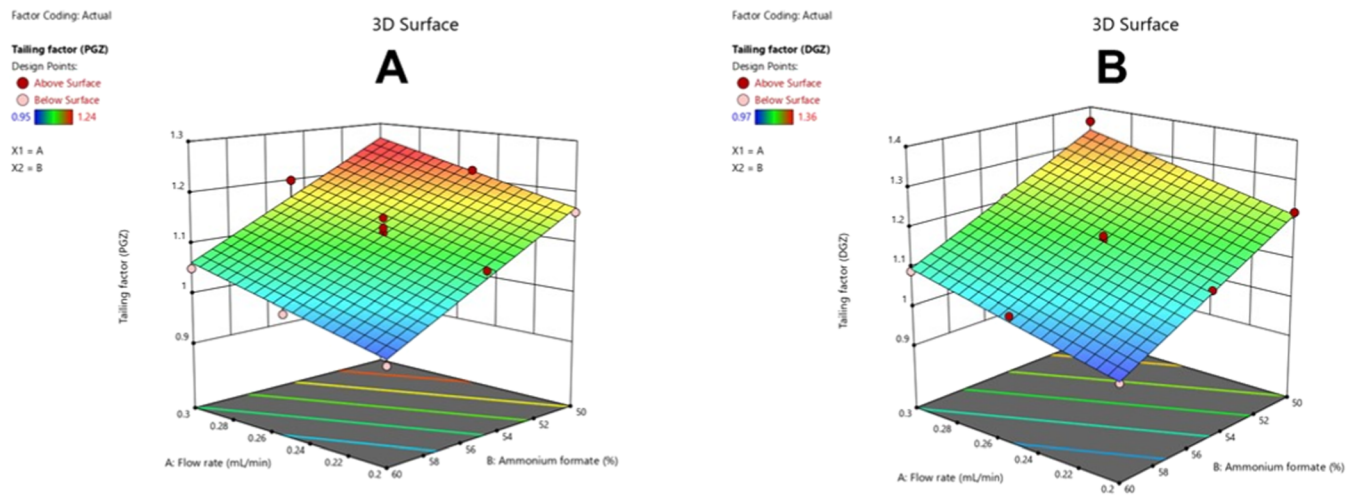

Figure 3. Impact of the flow rate and percent of ammonium formate buffer in the mobile phase on the tailing factors of (A) PGZ and (B) DGZ.

3.2.3. Tailing Factor. The tailing factors of PGZ and DGZ peaks ranged from 0.95 to 1.24 min and from 0.97 to 1.36 min, respectively. Table 6 shows that both independent factors significantly influenced the measured tailing factors of PGZ and DGZ. The impact of the flow rate and the percent of ammonium formate on the tailing factor is shown in Figure 3A,B, while the magnitude of their impact can be estimated from the value of the factor coefficient in the polynomial equations (eqs 7 and 8). The flow rate positively impacted the value of the tailing factor with no significant influence. On the

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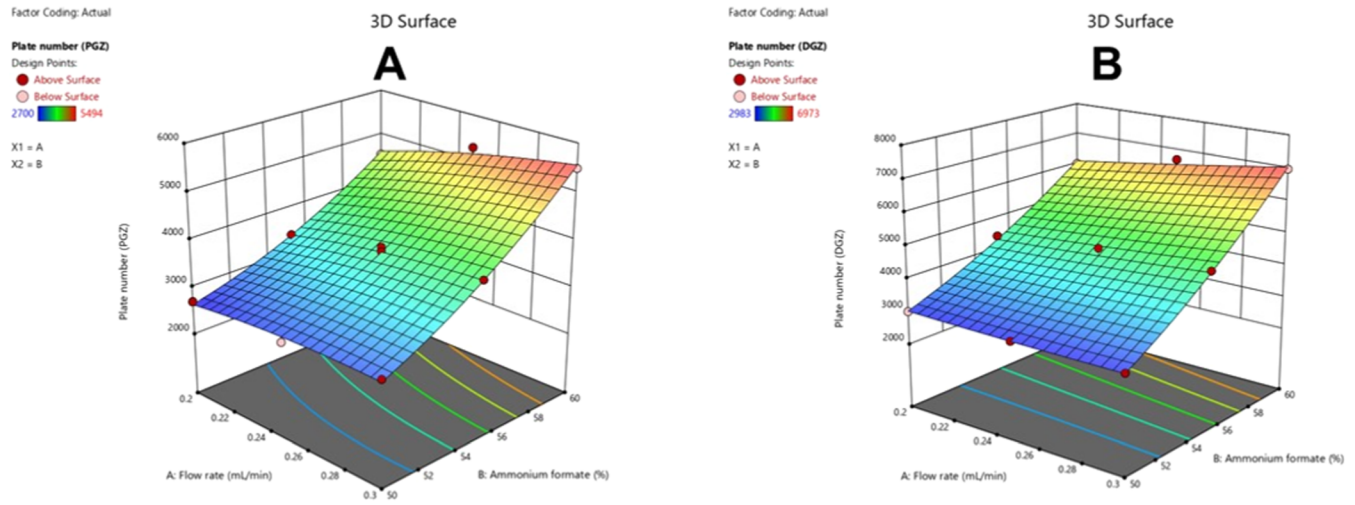

Figure 4. Impact of the flow rate and percent of ammonium formate buffer in the mobile phase on the plate numbers of (A) PGZ and (B) DGZ.

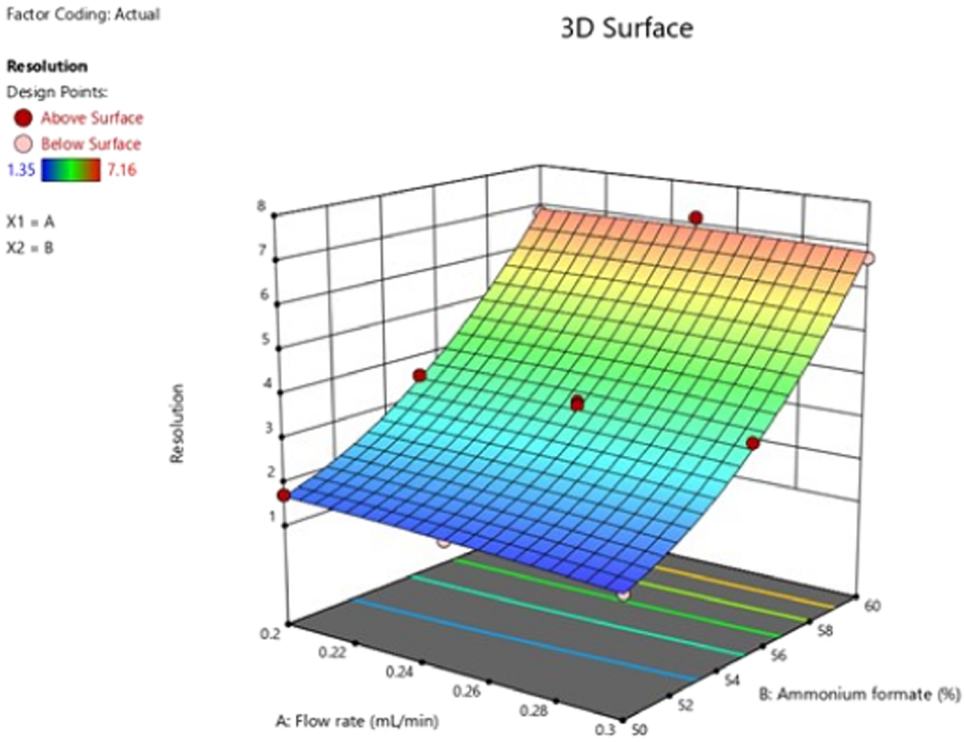

Figure 5. Impact of the flow rate and percent of ammonium formate buffer in the mobile phase on the resolution of PGZ and DGZ peaks.

contrary, decreasing the percentage of ammonium formate significantly increases the tailing factor of the drug's peaks.

$$\text{tailing factor (PGZ)} = 1.12 + 0.05\ \text{flow rate} - 0.10\%\ \text{ammonium formate} \quad (7)$$

$$\text{tailing factor (PGZ)} = 1.16 + 0.06\ \text{flow rate} - 0.12\%\ \text{ammonium formate} \quad (8)$$

The tailing factor of the detected eluted peak is considered an important parameter during the optimization of the UPLC method. It reflects the symmetric elution of the analyte during elution when its value is close to one. The ratio of ammonium formate in the mobile phase significantly influences the tailing factor of the detected peak. This could be attributed to the impact of the mobile phase composition and the ratio of ammonium formate buffer on the ionization of both drugs.[37] In addition, Alshora et al. found that decreasing the aqueous phase ratio resulted in a significant enhancement in peak symmetry.[38] This could be attributed to the uniform flow of analytes with columns during elution and the reduction of the tailing factor of the detected peak.

3.2.4. Plate Number. The calculated plate numbers of the PGZ and DGZ peaks ranged from 2700 to 5494 and from 2983 to 6973, respectively. ANOVA analysis revealed that the flow rate and percent of ammonium formate buffer in the mobile phase significantly affected the plate number of PGZ

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and DGZ (Table 6). The impact of two independent factors on the measured retention time is shown in Figure 4A,B. However, the magnitude of each variable could be estimated from the numerical value in the polynomial equations (eqs 9 and 10). Overall, increasing the flow rate and percent of ammonium formate buffer in the mobile phase resulted in a significant increase in the measured plate number with the pronounced impact of the latter.

$$\begin{aligned}\text{plate number (PGZ)} &= 3761.10 + 279.33\ \text{flow rate} + 1180.83\%\ \text{ammonium formate} + 189.00 \times \text{flow rate} \times \%\ \text{ammonium formate} - 85.86\ \text{flow rate}^2 + 236.64\%\ \text{ammonium formate}^2 \quad (9)\end{aligned}$$

$$\begin{aligned}\text{plate number (DGZ)} &= 4507.69 + 221.00\ \text{flow rate} + 1782.67\%\ \text{ammonium formate} + 245.50 \times \text{flow rate} \times \%\ \text{ammonium formate} + 24.59\ \text{flow rate}^2 + 254.59\%\ \text{ammonium formate}^2 \quad (10)\end{aligned}$$

Lowering the flow rate and increasing the ratio of the aqueous buffer can enhance the plate number in chromatography. This is because decreasing the flow rate allows for longer interactions between the analytes and the stationary phase. This leads to better separation and increases the peak area. This, in alignment with the previous reported study, showed that a low flow rate resulted in a peak with a high theoretical plate count.[31] Additionally, increasing the ratio of the aqueous buffer can improve the separation efficiency by promoting stronger interactions between the analytes and the stationary phase. This is achieved through increasing the retention time and results in narrower peaks and higher plate numbers.

3.2.5. Resolution of the Two Drug Peaks. The resolution of PGZ and PGZ peaks from each other ranged from 1.35 to 7.16. Table 6 shows that the flow rate has no significant influence on the peak resolution, while the percentage of ammonium formate significantly affects the peak resolution. The influence of two independent factors on the peak's resolution is shown in Figure 5, while the magnitude of their impact can be estimated from eq 11. The numerical value of the percent of ammonium formate buffer indicates its significant influence on the resolution of drug peaks, and the positive sign indicates its agonistic effect on the peaks' resolution.

$$\begin{aligned}\text{resolution} &= 3.43 - 0.09\ \text{flow rate} + 2.68\%\ \text{ammonium formate} + 0.07 \times \text{flow rate} \times \%\ \text{ammonium formate} - 0.05\ \text{flow rate}^2 + 0.82\%\ \text{ammonium formate}^2 \quad (11)\end{aligned}$$

3.3. Optimization of the UPLC Method. DoE software selected an optimized UPLC method condition with maximum desirability based on the following criteria: minimum retention time, maximum peak area, tailing factor close to 1, plate number above 2000, and resolution value of more than 2. These parameters ensure that the system is suitable for the optimized method. Reduction in the retention time of drugs reduces the consumption of the mobile phase and saves time via reducing the total run time.[39] In addition, maximization of the peak area could significantly improve the sensitivity and accuracy of the developed method.[40] Furthermore, the tailing factor and plate numbers are measured to ensure peak symmetry and column efficiency, respectively.[41] Figure 6

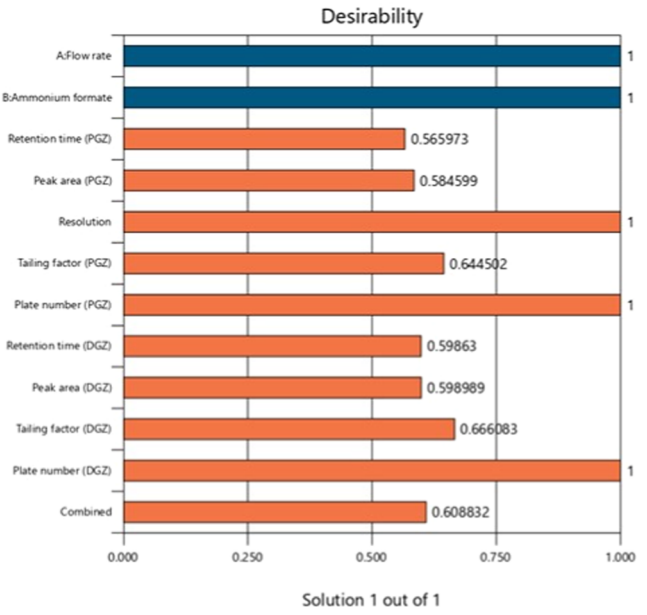

Figure 6. Bar graph representing the expected desirability of responses based on the requested constraints.

shows the optimized conditions with the desirability of chromatographic response parameters to the selected criteria. The optimized UPLC method conditions are expected to be achieved with a 0.2464 mobile phase flow rate composed of 56.3% ammonium formate buffer.

The optimized method was set up on a UPLC instrument to measure analytical response parameters, which are presented in Table 7. Figure 7A,B show chromatograms obtained from the injected blank and standard solution. The developed method was able to resolve two peaks of drugs from the nearest background peaks, indicating the specificity and selectivity of the developed method. The retention times of PGZ and DGZ were 4.62 and 6.21 min, respectively. The corresponding peak areas were 537 and 6.68 mAU*min. Regarding system suitability, the calculated plate numbers were 4157 for PGZ and 5154 for DGZ, while the tailing factors for both peaks were found to be 1.11 and 1.12, respectively. Furthermore, the peaks of the two drugs were well resolved, with a resolution value of 4.57. The results listed in Table 7 demonstrate that the developed method adheres to system suitability parameters, with a plate number above 2000, a tailing factor less than 2, and peak resolution values greater than 2. It is clear from Table 7 that the measured analytical response parameters fit within 95% of the prediction intervals. This indicates the validation of the design experiment to predict analytical response parameters at the studied range of independent responses.

3.4. Validation of the UPLC Method. Figure 8A,B shows a linear calibration curve with regression coefficient values of 0.9996 and 0.9991 over the concentration range 1.0−50.0 μg/mL for PGZ and DGZ, respectively. The equation obtained from the plotting of the theoretical concentration against the

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Table 7. Validation of the Designed Experiment Based on the Suggested Condition for the UPLC Method

measured response		predicted mean	SD	N	SE pred	95% PI low	data mean	95% PI high
retention time	PGZ	4.70	0.06	3	0.04	4.60	4.62	4.80
	DGZ	6.07	0.09	3	0.06	5.92	6.05	6.21
peak area	PGZ	5.21	0.15	3	0.11	4.96	5.34	5.47
	DGZ	6.31	0.26	3	0.17	5.94	6.12	6.68
tailing factor	PGZ	1.09	0.02	3	0.0143166	1.05	1.11	1.12
	DGZ	1.12	0.02	3	0.0110755	1.10	1.12	1.14
plate number	PGZ	4055.07	105.59	3	75.00	3877.73	4157	4232.41
	DGZ	4960.71	149.64	3	106.283	4709.39	5154	5212.03
peak resolution		4.17	0.23	3	0.166825	3.78	4.57	4.57

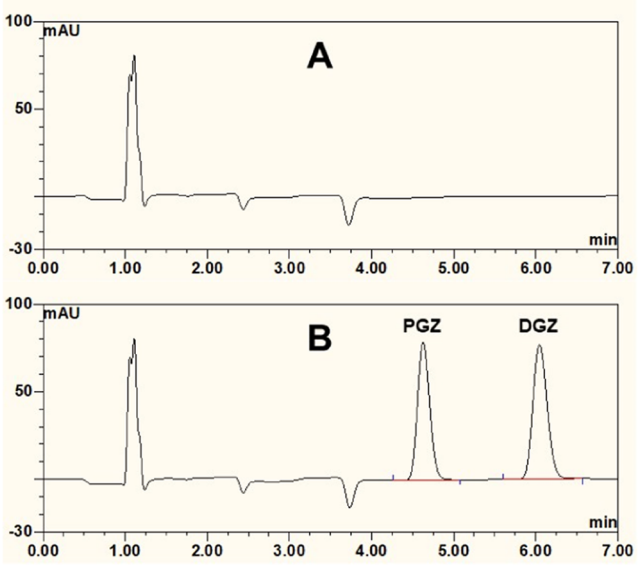

Figure 7. Chromatogram of the injected (A) blank solution (methanol) and (B) standard solution containing PGZ and DGZ.

peak area of PGZ ($y = 0.3198 \times x + 0.0518$) and DGZ ($y = 0.3628 \times x - 0.058$) would be utilized to determine the actual concentration of both drugs (Table 8). The calculated QL values for PGZ and DGZ were 3.5 and 2.5 μg/mL, respectively.

The assumption of homoscedasticity was tested for the regression of both curves. Table 9 shows that the Pearson correlations were calculated to be −0.009 and 0.165 for PGZ and DGZ, respectively. The low Pearson correlation coefficients indicate a very weak relationship between the absolute residuals and predicted values. The p-values were >0.05 for both drugs (PGZ: p-value = 0.984; DGZ: p-value = 0.724), suggesting that these correlations are not statistically significant and the homoscedasticity assumption was satisfied. This means that the variability in measurements is consistent across the range of analyte concentrations. The scatter plots shown in Figure 9 visually confirm this lack of pattern in the

Table 8. Linear Regression Analysis of PGZ and DGZ via the Developed UPLC Method[a]

parameter	pioglitazone	dapagliflozin
range (μg/mL)	1−50	1−50
regression coefficient	0.9997	0.9998
slope	0.3198	0.3628
intercept	0.0518	−0.058
SD	0.113	0.096
DL (μg/mL)	1.2	0.9
QL (μg/mL)	3.5	2.6

[a]DL: detection limit and QL: quantification limit.

Table 9. Correlation Table for the Homoscedasticity Assumption for PGZ and DGZ

		PGZ		DGZ	
		ABS_RES1	PRED1	ABS_RES1	PRED1
ABS_RES1	Pearson correlation	1	−0.009	1	0.165
	sig. (2-tailed)		0.984		0.724
	N	7	7	7	7
PRED1	Pearson correlation	−0.009	1	0.165	1
	sig. (2-tailed)	0.984		0.724	
	N	7	7	7	7

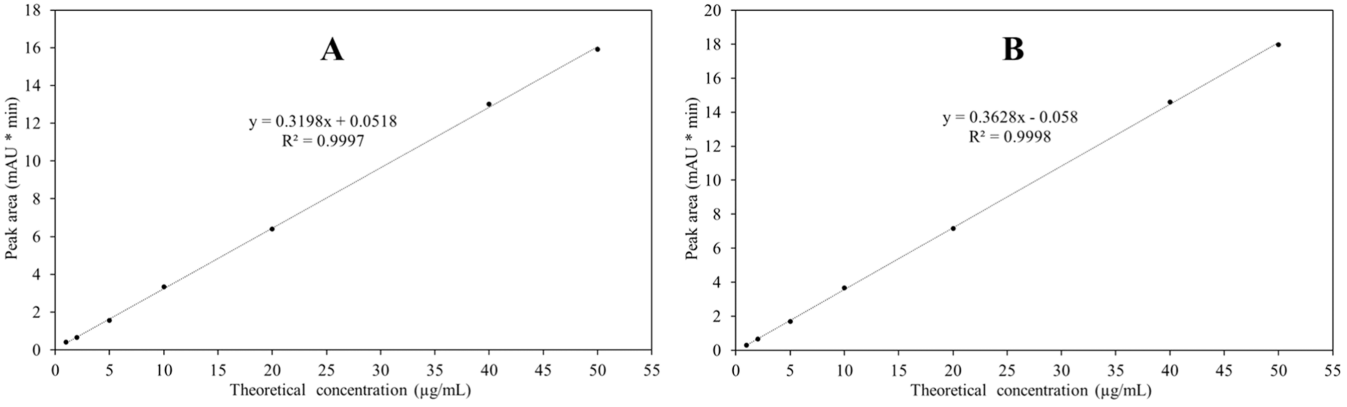

Figure 8. Calibration curves for (A) PGZ and (B) DGZ.

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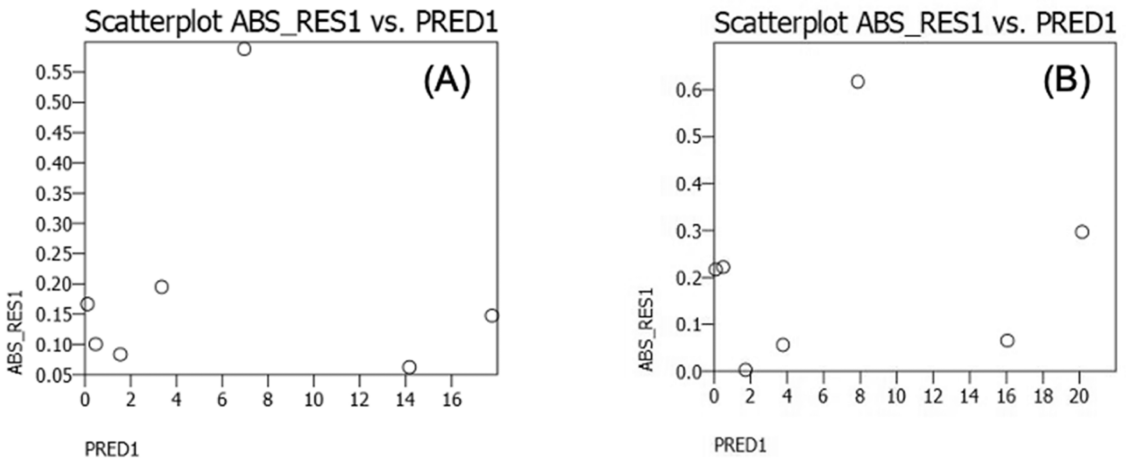

Figure 9. Scatter plots for the absolute residuals versus the predicted values for the bivariate correlation curves for (A) PGA and (B) DGZ.

residuals, further supporting the homoscedasticity assumption. This proves that the regression equation and the associated statistics assume that the amount of error or misprediction is equal across the entire continuum of the predictive equation. This demonstrates that our method is reliable and accurate across the entire concentration range, which is crucial for its application in quantifying PGZ and DGZ in pharmaceutical formulations.

The calculated values of accuracy (% recovery) and precision (% RSD) for the prepared quality control samples are presented in Table 10. It was found that the accuracy

Table 10. Intra- and Interday Precision for the Analysis of Pioglitazone and Dapagliflozin

	intraday accuracy and precision		interday accuracy and precision	
quality control sample	accuracy (%)	precision (% RSD)	accuracy (%)	precision (% RSD)
		PGZ		
3.5 μg/mL	108.98	5.56	109.25	2.86
10.0 μg/mL	101.56	1.82	100.21	0.56
20.0 μg/mL	95.54	1.33	92.45	0.79
40.0 μg/mL	99.15	0.76	99.29	3.38
		DGZ		
2.5 μg/mL	105.12	6.27	112.73	0.84
10.0 μg/mL	100.68	1.84	98.32	1.12
20.0 μg/mL	96.51	1.54	92.79	0.95
40.0 μg/mL	99.89	1.56	99.41	3.55

ranges of PGZ for intra- and interday samples were 95.54−108.98 and 92.45−109.25% and those of DGZ were 96.51−105.12 and 98.32−112.73%, respectively. However, the estimated intra- and interday precision ranges for PGZ were 0.76−5.56 and 0.56−3.38% RSD and those for DGZ were 1.54−6.27 and 0.84−3.55% RSD, respectively. The quantification limit was included in the accuracy assessment to ensure the method's reliability at the lower end of the analytical range. This is particularly important for our application, as it demonstrates the method's capability to accurately quantify the analytes at concentrations exactly at the lower limit of the validated range. The results obtained meet the acceptance criteria for the method performance established by the AOAC standards.

3.5. Assessing the Environmental Sustainability of the UPLC Method Developed Using AGREE Software.

AGREE, a newly developed tool for assessing greenness, stands out by utilizing the 12 principles of GAC as its input criterion and incorporating both qualitative and quantitative aspects. AGREE software can be obtained online for no cost by clicking on the following link: http://www.mostwiedzy.pl/AGREE. The calculator generates a graph that is easy for the user to understand and offers a thorough score. Compared to other software programs, such as NEMI and AES, this tool is widely regarded as the most helpful for assessing the environmental sustainability of analytical methods. It takes into account the environmental impact of these approaches, including their effects on the analyst and the sources of potential hazards.[42]

The sustainability assessment of the suggested UPLC approach's environmental impact, also referred to as "greenness," was carried out using in silico AGREE software. This program includes all 12 criteria set by the GAC organization.[22] The software assigns numerical values between 0.0 and 1.0 to many characteristics of the GAC system, creating analytical scales that measure the degree of environmental friendliness. The results are visually represented by a circular diagram that includes a wide range of colors, from red to dark green, representing twelve unique characteristics. Figure 10 illustrates the ecologically sustainable aspect of UPLC technology in use. The score of 0.77 was calculated based on an assessment of many qualities associated with the present methodology. The score serves as a measure for evaluating the degree of environmental sustainability achieved by UPLC technology.

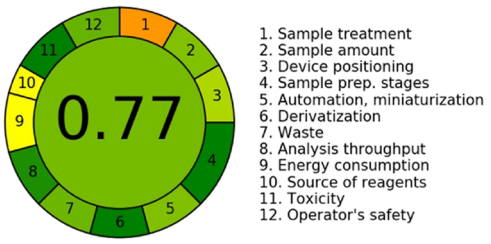

Figure 10. AGREE program was used to demonstrate the ecologically sustainable features of the developed UPLC. The outcomes are presented in a circular diagram with colors ranging from red (indicating low greenness) to dark green (indicating high greenness). The colors symbolize twelve distinct features.

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A score less than 1.0 signifies a higher level of ecological sustainability in the analytical procedure. The newly designed UPLC technology demonstrates a high level of sustainable development, with eco-scale values ranging from 0.75 to 1.00.

3.6. Application of the Developed UPLC Method. *3.6.1. Application on Marketed Tablets.* The current study showed that the developed method was able to determine the PGZ and DGZ concentrations within marketed tablets simultaneously. The PGZ and DGZ recovery percentages were 94.26% (±3.62%) and 101.16% (±4.04%), respectively. This result indicates the ability of the optimized UPLC method to estimate both drugs simultaneously and resolve them from the tablet matrix components.

3.6.2. Application on the Prepared SNEDDS Formulation. For further application, the SNEDDS formulation was prepared as a promising approach to enhance the bioavailability of PGZ and DGZ. The prepared SNEDDS formulation was loaded with PGZ and DGZ to prepare a drug-loaded SNEDDS formulation. After that, the drug concentration was measured using the developed UPLC method to ensure its ability to detect PGZ and DGZ within the proposed formulation. The results showed that the percentage of drug recovery was between 85.8 and 75.9%. Therefore, the present results show that entrapped drugs were not completely recovered from the loaded SNEDDS formulations. Therefore, the effect of the matrix components on drug extraction is examined in the next section.

3.6.3. Effect of the Matrix on Accuracy and Precision. Table 11 shows the accuracy and precision results. The results

Table 11. Measured Accuracy and Precision of the Effect of the Matrix

nominal concentration	accuracy (%)	precision (% RSD)
	PGZ	
3.5 μg/mL	96.7	2.84
10.0 μg/mL	96.7	0.93
40.0 μg/mL	96.5	0.87
	DGZ	
2.5 μg/mL	97.9	2.25
10.0 μg/mL	92.1	1.01
40.0 μg/mL	97.8	0.60

showed that the matrix had no significant effect on determining the drug concentration with acceptable recovery values. Although the matrix study showed no interference, it is possible that when the drug is actually loaded into the SNEDDS formulation, it interacts more strongly with the excipients. This could lead to incomplete extraction or reduced detection of the drug. Alternatively, the drug may be more soluble in the SNEDDS formulation than in the extraction solvent, leading to incomplete extraction when recovering the drug from the loaded formulation.

3.7. Future Prospective. Stability issues due to drug incorporation in the SNEDDS need to be addressed, as the drug might degrade or transform during the SNEDDS preparation process, resulting in a lower detectable amount.

4. CONCLUSIONS

In the present study, a validating UPLC method was successfully developed by utilizing advanced DoE software. It allows optimization of the methods with a lower retention time, higher peak area, and symmetric peaks with a high plate number of well-resolute peaks. The method was assessed in terms of linearity, accuracy, and precision, yielding exceptional results. This remarkable level of accuracy and precision allows for the precise and reliable measurement of drug doses in the proposed combination for the treatment of diabetes. The validated UPLC method holds great promise as a comprehensive and trustworthy analytical tool for future research and clinical applications in the ever-evolving field of diabetes treatment.

■ ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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Author Contributions

E.M.E.: Conceptualization, methodology, formal analysis, review, and editing. A.Y.S.: Conceptualization, methodology, writing—original draft preparation, review, and editing. M.W.A.: Methodology and writing—original draft preparation. M.A.A.: Review and editing. All authors have read and approved the final version of the manuscript.

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Notes

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