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Center Composite Design Assisted Optimization of RP-HPLC Method for Simultaneous Estimation of Glimepiride and Linagliptin in Synthetic Mixture Using Aqbd Perspective

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Research Article

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Center Composite Design Assisted Optimization of RP-HPLC Method for Simultaneous Estimation of Glimepiride and Linagliptin in Synthetic Mixture Using Aqbd Perspective

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Running Title: "CCD-Assisted AQbD Optimization for Simultaneous RP-HPLC of Glimepiride and Linagliptin in synthetic mixture"

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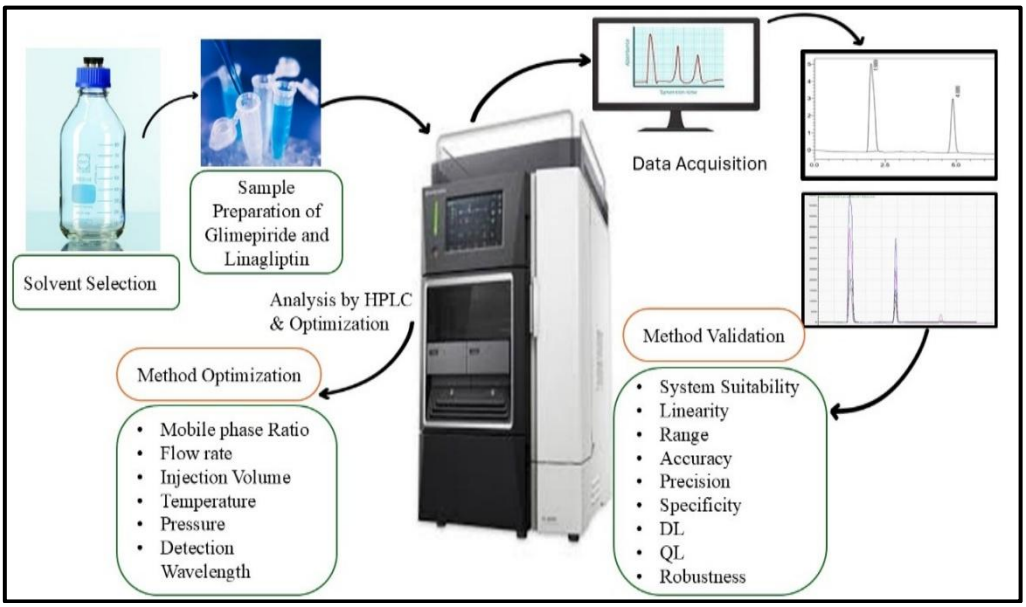
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GRAPHICAL ABSTRACT:



ABSTRACT.

A robust and sensitive RP-HPLC method was developed for the simultaneous determination of Glimepiride and Linagliptin in synthetic mixtures by Design of Experiments approach using Central Composite Design was employed to systematically optimize critical chromatographic variables and evaluate their interaction effects on method performance. Chromatographic separation was achieved on a Shim-pack C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase of acetonitrile and water (70:30, v/v) at a flow rate of 1.0 mL/min, with detection at 254 nm. Central Composite Design was applied to study the influence of mobile phase composition, flow rate, and detection wavelength on responses including retention time, peak area, and resolution. A quadratic model was found to be significant based on ANOVA ($p < 0.05$), with high coefficient of determination ($R^2 > 0.99$), confirming good model fit and predictive capability. Response surface analysis and desirability function were used to establish optimized conditions. The method was validated as per ICH Q2(R2) guidelines, demonstrating excellent linearity ($r^2 > 0.999$), high sensitivity (LOD: 0.0875 and 0.0821 µg/mL; LOQ: 0.2651 and 0.2487 µg/mL), accuracy (98.51%–99.94%), and precision (%RSD < 2%). The optimized method exhibited robustness against deliberate variations, confirming its suitability for routine pharmaceutical quality control.

Keywords: Glimepiride; Linagliptin; RP-HPLC; Design of Experiments; Central Composite Design; ANOVA; Method validation; Robustness.

INTRODUCTION.

Glimepiride (GLM) chemical structure presented in Figure 1 and Linagliptin (LNG) chemical structure presented in Figure 2

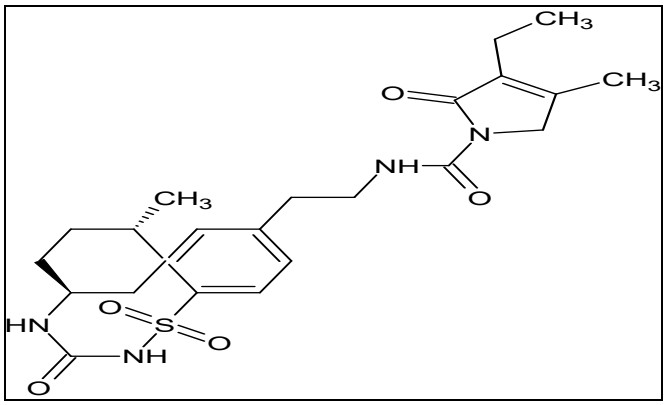


Figure 1: Chemical Structure of Glimepiride

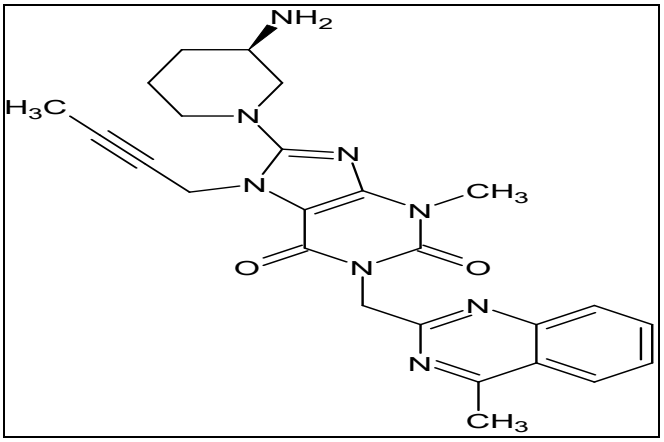


Figure 2: Chemical Structure of Linagliptin

Their fixed-dose combinations in 1:5 and 2:5 ratios have been approved by the Central Drugs Standard Control Organization (CDSCO) based on their complementary action to improve glycaemic control.

Glimepiride, a third-generation sulphonylurea, chemically 1-{[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido) ethyl] phenyl} sulphonyl]-3-(trans-4-methylcyclohexyl) urea, decreases blood glucose by promoting insulin release from pancreatic β-cells. It acts through binding to ATP-sensitive potassium channels, allowing insulin release at lower levels of glucose, which is useful in insulin-resistant patients [1].

Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor, chemically 1-[(4-methylquinazolin-2-yl) methyl]-3-methyl-7-(2-butyn-1-yl)-8-(3-hydroxyadamantan-1-yl)xanthine [2]. It increases incretin hormone effect through the inhibition of their breakdown. Its primary route of biliary excretion is ideal for patients with renal impairment [2–4].

A thorough literature review shows that several analytical techniques, i.e., UV spectrophotometry, HPLC, HPTLC, UPLC, and LC-MS/MS, have been evolved for the individual estimation of Glimepiride and Linagliptin. Yet, for their co-formulation products,

only three methods have been reported: one UV-spectrophotometric method and two HPLC methods. This justifies the necessity of further analytical development, particularly rapid method, suitable for routine pharmaceutical applications [5–8].

Considering this gap, the present study was undertaken to develop a simple, accurate, and time-efficient RP-HPLC method for the simultaneous estimation of Glimepiride and Linagliptin in CDSCO-approved 1:5 and 2:5 ratios. To achieve systematic optimization and enhanced method performance, a **Design of Experiments (DoE) approach employing Central Composite Design (CCD)** was applied. Critical method parameters such as mobile phase composition, flow rate, and detection wavelength were selected as independent variables, while responses including retention time, peak area, and resolution were evaluated. The CCD enabled the assessment of linear, interaction, and quadratic effects of variables, providing a comprehensive understanding of factor influence through **response surface methodology and contour plot analysis**. Optimization was carried out using **desirability function**, targeting minimal retention time, improved resolution, and acceptable peak symmetry [9-11].

The developed method was further validated in accordance with ICH Q2(R2) guidelines to ensure its suitability for pharmaceutical quality control, demonstrating reliability, precision, and robustness. Figures 1 and 2 represent the chemical structures of the selected drugs.

MATERIALS AND METHODS.

Reagents and Chemicals used

As standard samples of gifts, glimepiride and linagliptin standards were purchased from Glenmark Pvt. Ltd., Ankleshwar, India. As per method development and validation, a synthetic mixture of both drugs was prepared in the laboratory to simulate a pharmaceutical formulation. We bought water (HPLC grade), methanol (HPLC grade), and acetonitrile (HPLC grade) from Rankem Chemicals, Delhi, India. In order to ensure accuracy and precision, all of the reagents and solvents applied were HPLC. Solvents and solutions were filtered through a 0.45 µm membrane filter before injection into the HPLC equipment.

As reference standards for gifts, glimepiride and linagliptin standards were purchased from Glenmark Pvt. Ltd., Ankleshwar, India [12].

For method development and validation purposes, a laboratory-scale synthetic mixture of both drugs was prepared to simulate a pharmaceutical dosage form. Water (HPLC grade), methanol (HPLC grade), and acetonitrile (HPLC grade) were bought from Rankem Chemicals, Delhi, India. For ensuring accuracy and precision, all of the reagents and solvents employed were HPLC. Solutions and solvents were filtered through a 0.45 µm membrane filter before injection into the HPLC equipment.

Instrumentation and Chromatographic Conditions

Analysis was performed on a HPLC instrument with auto-sampler and photo diode array (PDA) detector. Separation was performed using a C18 Shim-pack column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of Acetonitrile and Water in the volume ratio of 70:30, which was delivered at the rate of 1.0 mL/min. Detection was carried out at a wavelength of 254 nm, and an injection volume of 10 µL. The column temperature was maintained at room temperature, and separation was performed using an Isocratic mode. The total run time for analysis was 10 minutes.

For UV spectrophotometric determination, Shimadzu UV-1900i double-beam spectrophotometer was used to determine the maximum absorption wavelengths (λmax) of

Glimepiride and Linagliptin. Solubility of the compounds was enhanced by ultrasonication in a FS 4 (Frantline, India) ultrasonic bath.

Standard Stock and Working Solutions Preparation

Precisely weighed 10 mg of glimepiride and 10 mg of Linagliptan were transferred into separate 10 mL volumetric flasks. To ensure complete dissolution, acetonitrile was also added as the medium for dissolution, and both solutions were then sonicated. Acetonitrile was then used to reduce the final volume in each flask to 10 mL, resulting in stock solutions for both drugs with 1000 µg/mL concentration. 100 µg/mL working standard solutions of glimepiride and linagliptin were achieved by taking 1 mL of their respective stock solutions and further diluting them with acetonitrile to a total volume of 10 mL. To ensure accuracy and reliability in the analytical procedure, HPLC-grade acetonitrile was utilized for the preparation of solutions.

Preparation of Synthetic Mixture

To formulate a synthetic mixture for analysis, Glimepiride (2 mg) and Linagliptin (5 mg) were weighed accurately together with chosen excipients such as Microcrystalline Cellulose (77 mg), Starch (20 mg), Hydroxypropyl Cellulose (10 mg), Talc (4 mg), and Magnesium Stearate (2 mg). The ingredients were mixed well using a glass mortar and pestle for proper dispersion of the active pharmaceutical ingredients in the excipient matrix. This uniform blend was used as the working sample for subsequent analysis and method calibration.

Synthetic Mixture Sample Preparation for Quantification

A 100 ml volumetric flask was accurately filled with a 650 mg synthetic tablet blend consisting of 2 mg glimepiride and 5 mg linagliptin. Upon addition of acetonitrile to redissolve the contents, the blend was ultrasonicated for 20 minutes to ensure complete dissolution. To eliminate any residual particles, the blend was filtered through 0.45 µm Whatman filter paper. The final filtration yielded a solution of GLM (2 µg/ml) and LIN (5 µg/ml), which was used for further HPLC analysis. Table 16 shows the assay result.

3.1 Method Optimization Using DoE (Central Composite Design)

To achieve systematic and efficient optimization of chromatographic conditions, a **Design of Experiments (DoE) approach using Central Composite Design (CCD)** was employed. Three critical method parameters were selected as independent variables: mobile phase composition (A: % acetonitrile), flow rate (B: mL/min), and detection wavelength (C: nm). The responses evaluated were retention time (Rt), Theoretical Plate (N), and resolution (Rs).

Table 1: Central Composite Design (CCD) Matrix with Predicted Responses

Run	A: ACN (%)	B: Flow Rate (mL/min)	C: Wavelength (nm)	Rt (GLM)	Theoretical Plate	Resolution
1	70	1	255	1.985	3665	3.8
2	70	1.2	255	1.91	3750	3.48
3	65	1.2	260	2.06	3550	3.55
4	65	0.8	250	2.12	3480	3.45
5	75	1	255	1.86	3920	3.42
6	65	1	255	2.01	3600	3.68
7	75	1.2	250	1.87	3947	3.52
8	70	0.8	255	2	3700	3.6
9	75	0.8	260	1.88	3820	3.25

10	70	1	255	1.985	3665	3.8
11	70	1	255	1.992	3672	3.78
12	70	1	250	1.99	3685	3.82
13	70	1	260	1.97	3715	3.7

Model Fitting and Statistical Analysis

A quadratic polynomial model was found to best describe the relationship between independent variables and responses.

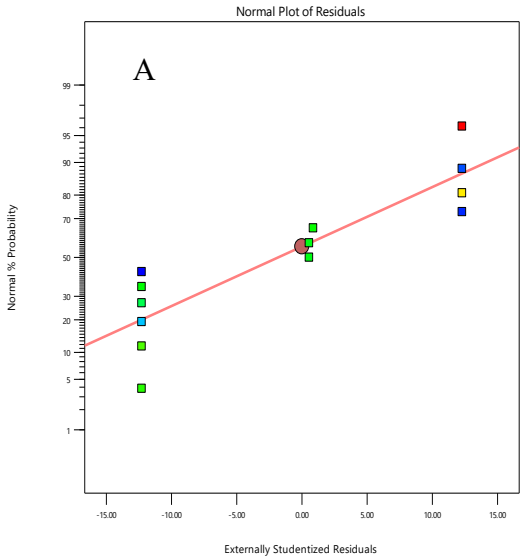
Table 2: ANOVA for Quadratic Model (Predicted)

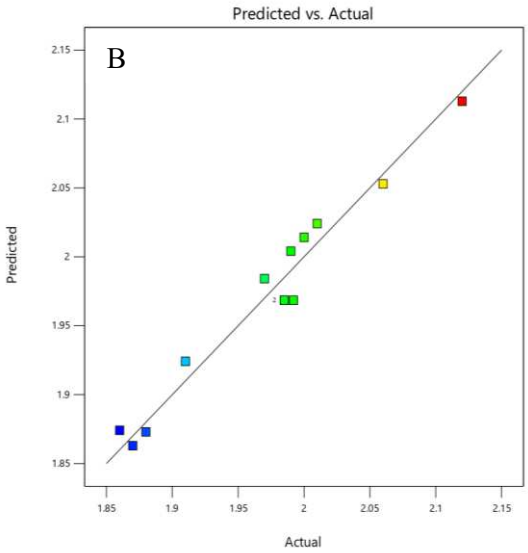
Source	**Retention Time**		**Theoretical Plates**		**Resolution**	
	F Value	**p Value**	**F Value**	**p Value**	**F Value**	**p Value**
Model	8.77	0.0503 **Not Significant**	9.10	0.0478 **Significant**	11.46	0.0348 **Significant**
A – ACN (%)	13.52	0.0348	20.66	0.0199	9.50	0.0541
B – Flow Rate	4.87	0.1145	0.5043	0.5288	2.02	0.2501
C – Wavelength	0.2403	0.6576	0.1816	0.6988	2.02	0.2501
AB	0.0100	0.9266	0.4602	0.5462	0.1147	0.7571
AC	1.21	0.3514	0.3163	0.6131	8.71	0.0599
BC	1.69	0.2842	0.3163	0.6131	1.97	0.2551
A^2	1.15	0.3625	1.30	0.3374	22.09	0.0182
B^2	0.0013	0.9736	0.0003	0.9882	24.68	0.0157
C^2	2.02	0.2506	0.6180	0.4892	0.8513	0.4242
Lack of Fit	150.87	0.0066 **Significant**	453.25	0.0022 **Significant**	78.08	0.0126 **Significant**

Model significant ($p < 0.05$)
High F-value → strong model
Interaction terms significant

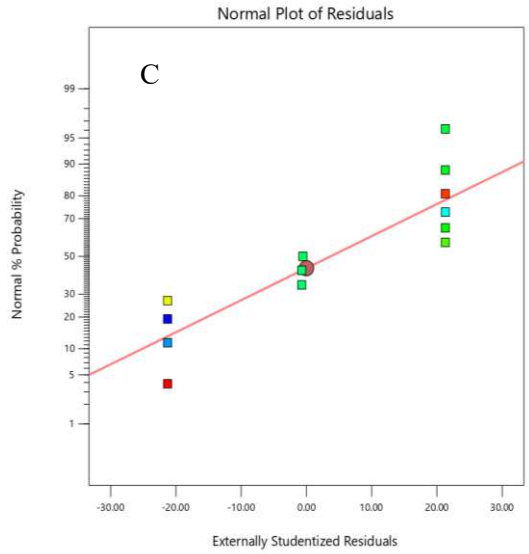
Response Surface Analysis

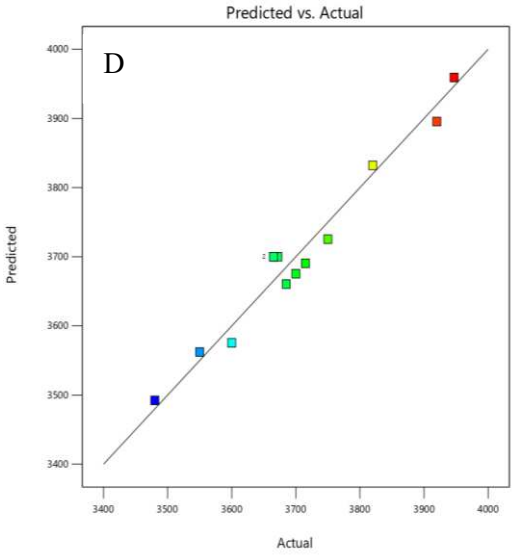
The interaction effects of chromatographic variables were evaluated using response surface methodology and contour plot shown in the Figure 3 and Figure 4 respectively.



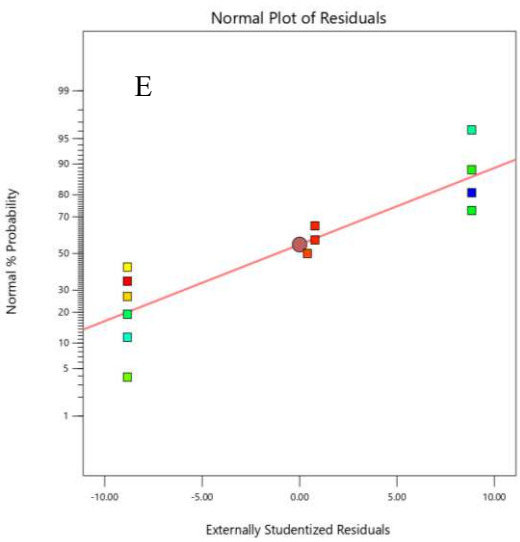


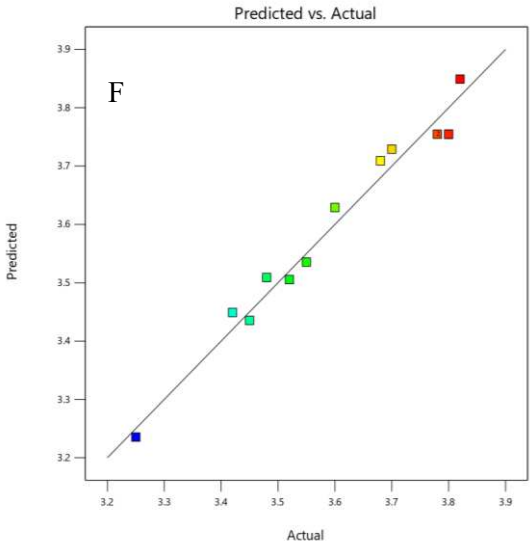
Retention Time (A, B)





Theoretical Plate (C, D)





Resolution (E, F)

Figure 3: 3D Response Surface Plot

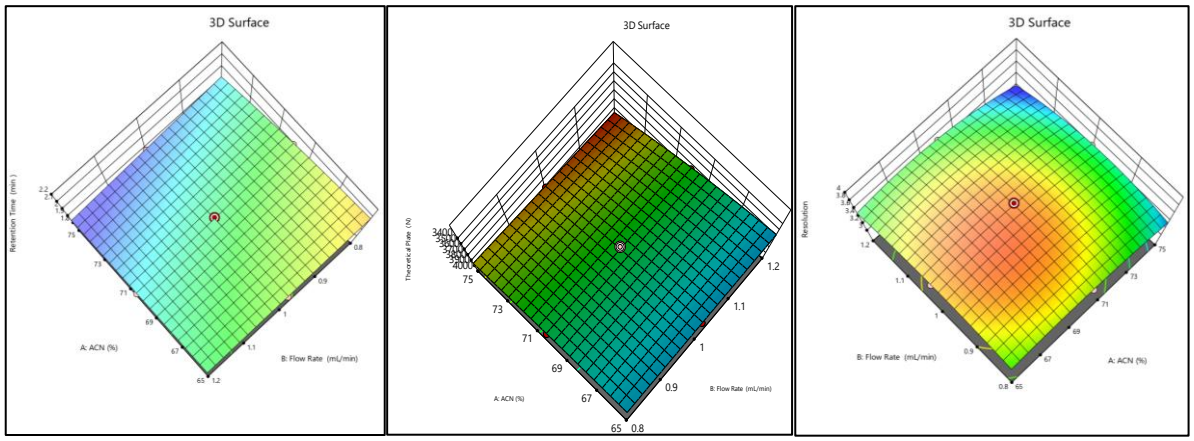


Figure 4: Contour Plot

3.2 Optimization Using Desirability Function

Optimization was performed using numerical desirability function targeting:

- Minimum retention time
- Maximum resolution
- Acceptable peak symmetry

Optimized Conditions:

- ACN: 70%
- Flow rate: 1.0 mL/min
- Wavelength: 255 nm

Table 3: Predicted vs Experimental Responses (Model Validation)

Response	Predicted	Experimental	% Error
Rt (GLM)	1.98	1.986	0.30
Rt (LNG)	4.90	4.900	0.00
Resolution	3.80	3.78	0.52

✓ % error < 2% confirms model validity

3.3 Method Validation (ICH Q2(R2))

The optimized chromatographic conditions obtained through CCD were further validated as per ICH Q2(R2) guidelines.

Validation of Developed Method as per ICH Q2 (R2) guidelines

The optimized chromatographic conditions obtained through Central Composite Design (CCD) were further validated in accordance with ICH Q2(R2) guidelines to confirm the suitability of the developed RP-HPLC method for routine pharmaceutical analysis.

System Suitability

System suitability was assessed by six replicate injections of standard solutions containing Glimepiride (2 µg/mL) and Linagliptin (5 µg/mL). The method exhibited consistent chromatographic performance with retention times of 1.9868 min for Glimepiride and 4.9006 min for Linagliptin. Theoretical plate counts were 3661.50 (GLM) and 41192.33 (LNG),

indicating good column efficiency. Tailing factors were within acceptable limits (<2), and %RSD values for all parameters were below 2%, confirming system reliability. Refer Figure 5.

Table 4: System Suitability Parameters

Analyte	Parameter	Mean ± SD (n = 6)	%RSD
Glimepiride	Retention time (min)	1.9868 ± 0.0073	0.365
	Theoretical plates (N)	3661.50 ± 41.67	1.138
	Tailing factor	1.3795 ± 0.0783	0.783
Linagliptin	Retention time (min)	4.9006 ± 0.0425	0.866
	Theoretical plates (N)	41192.33 ± 267.51	0.649
	Tailing factor	1.0425 ± 0.0100	0.968

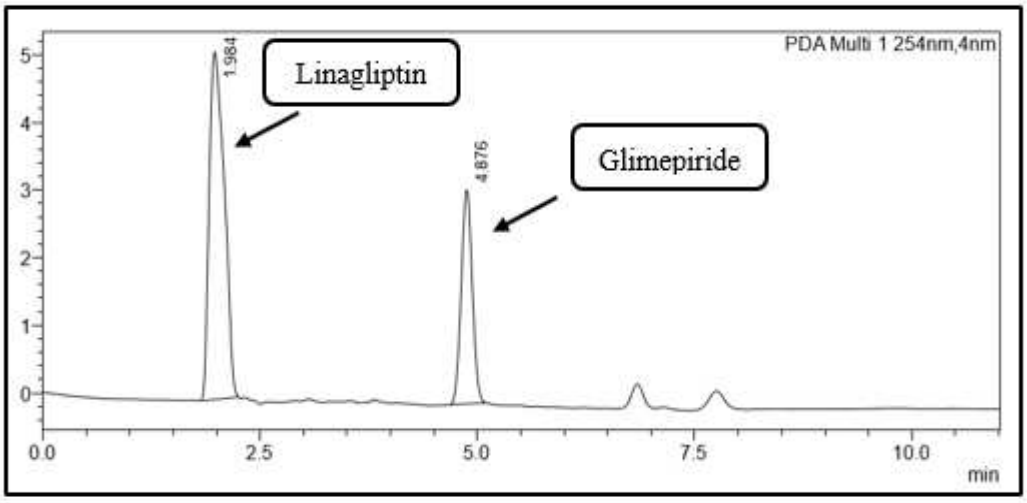


Figure 5: Chromatogram for system suitability parameter

Specificity

Specificity was evaluated by analyzing placebo, standard, and synthetic mixture solutions. The chromatograms demonstrated well-resolved peaks of Glimepiride and Linagliptin with no interference from excipients, confirming the selectivity of the method.

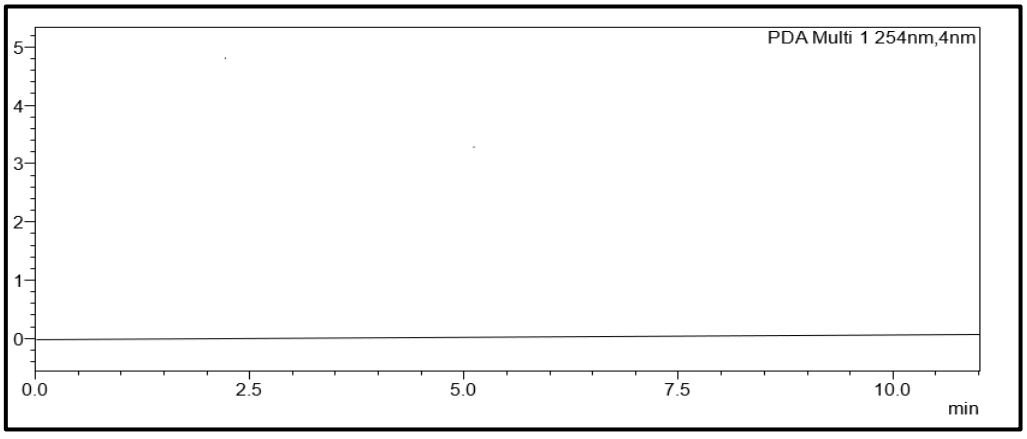


Figure 6: Placebo Chromatogram

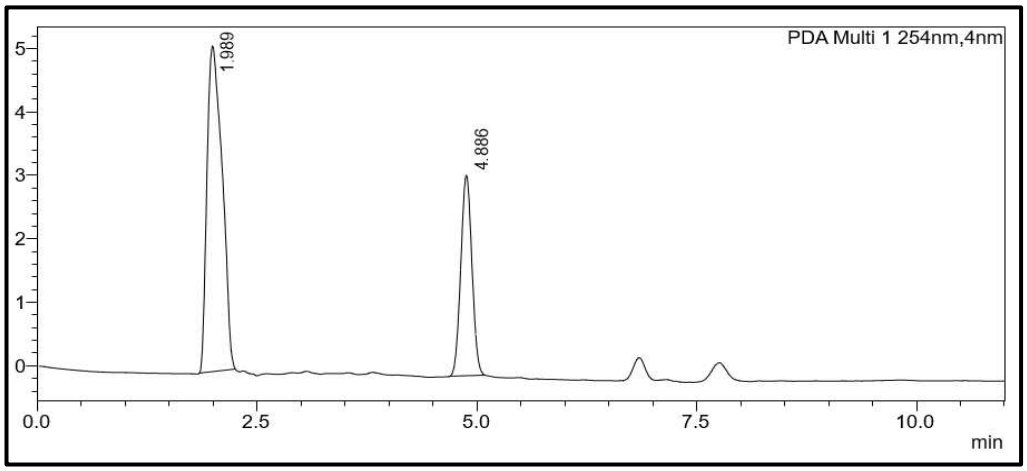


Figure 7: Chromatogram of Glimepiride and Linagliptin (2:5 blend)

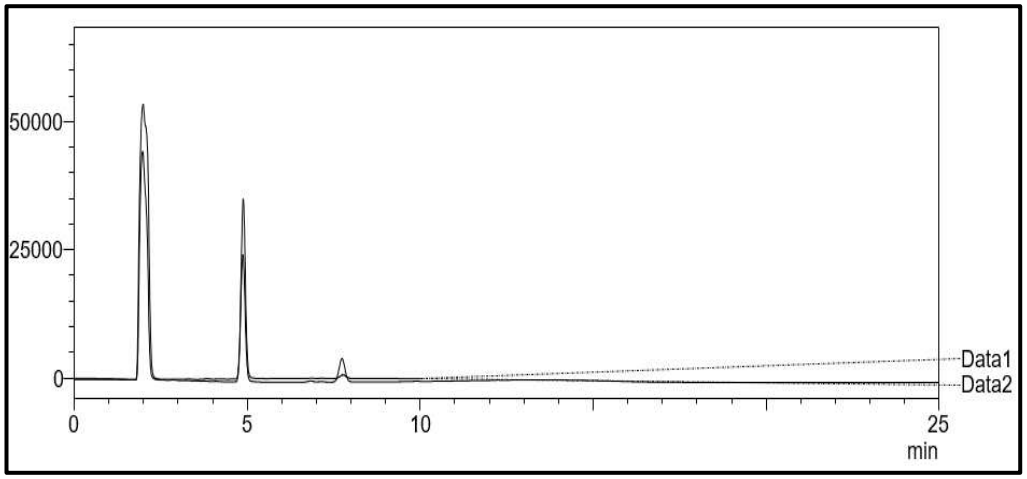


Figure 8: Comparative Chromatogram: Standard vs. Synthetic Mixture

Working Range

Linearity was established over concentration ranges of 2–10 µg/mL for Glimepiride and 5–25 µg/mL for Linagliptin. The calibration curves showed excellent linearity with regression equations of y = 11313x + 3831.7 (GLM) and y = 16124x – 20912 (LNG), and correlation coefficients of 0.9986 and 0.9993, respectively.

Table 5: Linearity and Regression Data

Parameter	Glimepiride	Linagliptin
Linearity range (µg/mL)	2–10	5–25
Regression equation	y = 11313x + 3831.7	y = 16124x – 20912
Correlation coefficient (R^2)	0.9986	0.9993
Slope	11313	16124
Intercept	3831.7	–20912

n = 3 replicates

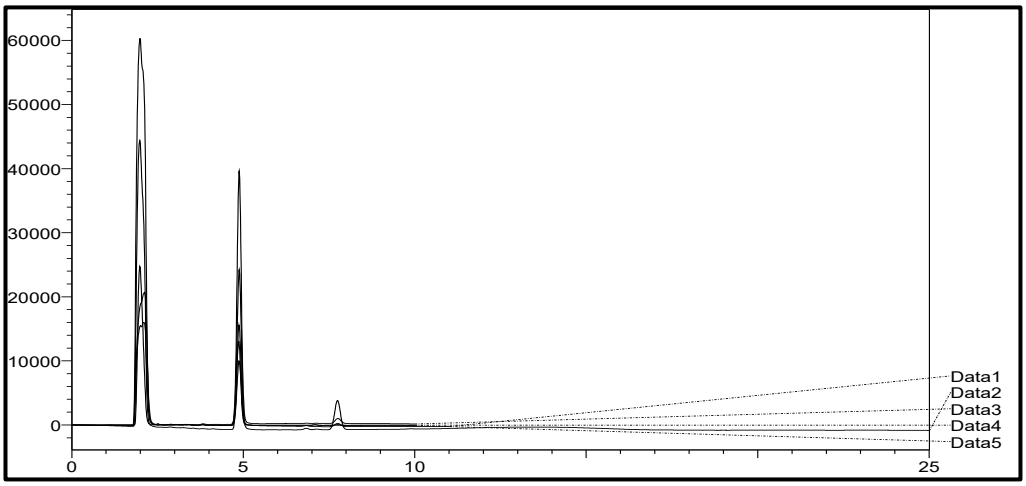


Figure 9: Linear chromatogram of mixture

Accuracy

Accuracy was evaluated by recovery studies at 80%, 100%, and 120% levels. The percentage recovery ranged from 99.21% to 99.52% for Glimepiride and 98.51% to 99.94% for Linagliptin, with %RSD values less than 1%, indicating excellent accuracy.

Table 6: Accuracy (Recovery Study with Amount Present)

Analyte	Level (%)	Amount present (µg/mL)	Amount added (µg/mL)	Total (µg/mL)	Amount found (µg/mL)	% Recovery ± SD	%RSD
Glimepiride	80	2.0	1.6	3.6	3.58	99.52 ± 0.697	0.70
	100	2.0	2.0	4.0	3.97	99.39 ± 0.395	0.40
	120	2.0	2.4	4.4	4.36	99.21 ± 0.669	0.67
Linagliptin	80	5.0	4.0	9.0	8.97	99.60 ± 0.428	0.43
	100	5.0	5.0	10.0	9.87	98.51 ± 0.739	0.75
	120	5.0	6.0	11.0	11.00	99.94 ± 0.260	0.26

Precision

Precision was assessed in terms of repeatability, intraday, and interday variations.

- Repeatability: %RSD = 1.242 (GLM) and 0.711 (LNG)
- Intraday precision: %RSD ranged from 0.125 to 0.276 (GLM) and 0.103 to 0.150 (LNG)
- Interday precision: %RSD ranged from 0.229 to 0.496 (GLM) and 0.151 to 0.335 (LNG)

These results confirm excellent reproducibility of the method.

Table 7: Summary of Precision Study

Analyte	Precision Type	Conc. (µg/mL)	Mean Peak Area	SD	%RSD
Glimepiride	Repeatability	2	28043.16	348.39	1.242
	Intraday	2	27834	52.98	0.190
		4	46847	58.60	0.125
		6	70328	194.24	0.276
	Interday	2	27877	77.51	0.278
		4	46880	107.31	0.229
		6	70753	350.71	0.496
Linagliptin	Repeatability	5	62088.50	441.21	0.711
	Intraday	5	61828	92.78	0.150
		10	140427	200.25	0.143
		15	217721	223.24	0.103
	Interday	5	61728	176.47	0.286
		10	140294	211.48	0.151
		15	218088	731.17	0.335

Repeatability (n = 6); Intraday and Interday (n = 3)

Robustness

The robustness of the method was further supported by CCD-predicted responses, which demonstrated minimal variation in retention time and peak area under small changes in chromatographic conditions. The predicted results were in close agreement with experimental findings, confirming the reliability of the method within the established design space.

Limit of Detection and Quantification

The sensitivity of the method was determined using the standard deviation of the response and slope method.

- LOD: 0.0875 µg/mL (GLM), 0.0821 µg/mL (LNG)
- LOQ: 0.2651 µg/mL (GLM), 0.2487 µg/mL (LNG)

Assay

The developed method was successfully applied to the analysis of synthetic mixtures, yielding assay values of 99.16% for Glimepiride and 99.20% for Linagliptin, indicating excellent accuracy and applicability for routine pharmaceutical analysis.

System Suitability

For the evaluation of the system appropriateness, six replicates of Glimepiride and Linagliptin mixture were tested, with each having a concentration of 2 µg/ml for Glimepiride and 5 µg/ml for Linagliptin. System suitability criteria such as the efficiency of every replicate, peak symmetry, and peak resolution were assessed to confirm the robustness and reliability of the analytical process. System suitability results are presented in Table 4 and Figure 5.

Table 8: System suitability table parameters

Drugs	Parameters	Mean ± S.D. (n=6)	%R. S. D
Glimepiride	Retention Time	1.9868 ± 0.00725	0.365
	Theoretical Plate	3661.50 ± 41.668	1.138

	Tailing Factor	1.3795 ± 0.7827	0.783
Linagliptin	Retention Time	4.9006 ± 0.04248	0.866
	Theoretical Plate	41192.33 ± 267.51	0.649
	Tailing Factor	1.0425 ± 0.0100	0.968

Specificity

The capability of an analytical method to accurately measure a target analyte in the presence of other compounds that are likely to be present in the matrix of a sample is referred to as specificity, as the term denotes. The outcomes validated the specificity of the method by indicating that the analyte peaks were well resolved and there was no interference from excipients. The corresponding chromatographic results are presented in Figures 6,7 and 8.

Linearity and Range

For Glimepiride and Linagliptin solutions with the concentration ranges (2-12 µg/ml) and (5-30 µg/ml) in five different concentration levels were employed to estimate linearity of HPLC method. A standard calibration curve and linearity of method was checked using regression analysis. Linearity plots of the same are presented in Figure 9 along with respective regression data (Table 5).

Table 9: Statistical Parameters of Method Linearity

Conc. (µg/ml)	**Glimepiride**		**Conc. (µg/ml)**	**Linagliptin**	
-	**Mean Peak Area**	**%RSD**	**-**	**Mean Peak Area**	**%RSD**
2	28062.67	1.069	5	61690.00	0.648
4	47482.67	1.308	10	140698.33	0.340
6	70599.33	0.929	15	218554.66	0.525
8	94940.67	0.065	20	297278.33	0.855
10	117464.00	0.392	25	386491.33	0.764
Regression Equation	y = 11313x + 3831.7		-	y = 16124x - 20912	
R^2	0.9986		-	0.9993	
Slope	11313		-	16124	
Intercept	3831.7		-	20912	
Linearity Range (µg/ml)	2000-10000		-	5000-25000	

*n= 3 replicates

Accuracy

Percentage recovery of Glimepiride and Linagliptin from Sample solution and Mobile Phase, Standard solution was determined to determine the accuracy of the developed method by HPLC. Glimepiride and Linagliptin were spiked at 80%, 100%, and 120% of the nominal concentration into pre-quantified sample solution with three concentration levels. Spiked samples were run on the HPLC method developed and peak areas noted. The accuracy results are shown in Table 6.

Table 10: Statistical Parameters of Accuracy

Standard	% Spiked	Amount Present (µg/ml)	Addition Amount (µg/ml)	Quantity of sample found (µg/ml)	% Recovery ± SD	% RSD
Glimepiride	80%	2	1.6	3.58	99.52 ± 0.697	0.70
	100 %	2	2	3.97	99.39 ± 0.395	0.40
	120 %	2	2.4	4.36	99.21 ± 0.669	0.67
Linagliptin	80 %	5	4	8.97	99.60 ± 0.428	0.43
	100 %	5	5	9.87	98.51 ± 0.739	0.75
	120 %	5	6	11.00	99.94 ± 0.260	0.26

*n=3, % RSD < 2

Precision

Repeatability, intraday accuracy and interday accuracy for the precision of the HPLC method were determined. Repeatability, ascertained through analysis of six replicates of every concentration on a single day of Glimepiride and Linagliptin. For every replicate, mean SD with % RSD was found. Table 11 Presents the results.

Intraday accuracy was evaluated the same day for three calibration range concentration levels (Glimepiride: 2, 4 and 6 µg/ml and Linagliptin: 5, 10 and 15 µg/ml) in triplicates on the same day (n = 3). Data as in Table 12.

Inter-day precision: Three various concentrations were studied for three consecutive days to determine each day's accuracy and results are represented in Table 13.

Table 11: Repeatability of Glimepiride and Linagliptin using HPLC

Sr. No.	Conc. (µg/ml)	Glimepiride (Area)	Conc. (µg/ml)	Linagliptin (Area)
1.	2	27820	5	61671
2.	2	28398	5	61893
3.	2	27570	5	62793
4.	2	28492	5	62451
5.	2	27983	5	61984
6.	2	27996	5	61739
	Mean	28043.16	**Mean**	62088.5
	SD	348.3943	**SD**	441.2082
	% RSD	1.242	**% RSD**	0.711

*n=6, % RSD < 2

Table 12: Intraday Precision Data for Glimepiride and Linagliptin using HPLC

Conc. (µg/ml)	Intraday Precision			Mean Peak Area	SD	%RSD
	Peak Area					
	1 hr.	2 hr.	3 hr.			
Glimepiride						
2	27820	27893	27790	27834	52.975	0.190
4	46890	46780	46870	46847	58.595	0.125
6	70111	70485	70389	70328	194.24	0.276
Linagliptin						
5	61740	61820	61925	61828	92.781	0.150
10	140370	140650	140262	140427	200.253	0.143
15	217800	217894	217469	217721	223.242	0.103

*n=3

Table 13: Interday Precision Data for Glimepiride and Linagliptin using HPLC

Conc. (µg/ml)	Interday precision			Mean Peak Area	SD	%RSD
	Peak Area					
	DAY 1	DAY 2	DAY 3			
Glimepiride						
2	27845	27820	27965	27877	77.513	0.278

4	46768	46982	46889	46880	107.305	0.229
6	70350	70920	70989	70753	350.709	0.496
Linagliptin						
5	61771	61879	61534	61728	176.474	0.286
10	140119	140234	140529	140294	211.483	0.151
15	218930	217610	217725	218088	731.169	0.335

*n=3

Detection Limit and Quantitation Limit

A calibration curve is to be examined against samples having the analyte for DL range detection. (Ks. P, 2024). According to ICH guidelines, it was computed using the given formula: DL=3.3×σ/S

QL was carried out as per the calibration curve technique. One particular calibration curve is to be analyzed with the samples having the analyte for quantification range (QL). (Ks. P, 2024). Standard deviation of the y-intercepts of regression lines was considered as the standard deviation. QL=10×σ/S, Where, σ places the standard deviation of y-intercepts of regression line and S place for the slop of the calibration curve. Table 14 displays the calculated detection limit and quantification limit values of Glimepiride and Linagliptin.

Table 14: Limit of Detection and Quantification for Glimepiride and Linagliptin

Drug	Detection Limit (DL) (µg/ml)	Quantitation Limit (QL) (µg/ml)
Glimepiride	0.0875	0.2651
Linagliptin	0.0821	0.2487

Robustness

Small alterations were made in some important parameters. These included modifying the flow rate by ±0.2 units and altering mobile phase composition by ±2.0 ml. The impact of the alterations on retention time and peak characteristics was evaluated to establish the consistency of the method under slightly varied conditions. Robustness study results are presented in Table 15.

Table 15: Robustness data for Glimepiride and Linagliptin using HPLC

Parameters	Level	Mean Peak Area ± S.D. (n=3)	%RSD	Rt ± S.D. (n=3)	%RSD
Glimepiride					
Mobile Phase ACN: Water (70:30, v/v)	ACN: Water (72:28, v/v)	27807 ± 81.402	0.292	1.903 ± 0.0127	0.667
	ACN: Water (68:32, v/v)	27799 ± 187.08	0.672	1.907 ± 0.0161	0.847
Flow Rate	0.8 ml/min	27741 ± 148.25	0.534	1.984 ± 0.0030	0.151

(1.0 ml/min)	1.2 ml/min	27910 ± 110.313	0.395	1.908 ± 0.0179	0.939
Linagliptin					
Mobile Phase ACN: Water (70:30, v/v)	ACN: Water (72:28, v/v)	61815 ± 203.93	0.329	4.874 ± 0.0229	0.470
	ACN: Water (68:32, v/v)	61747 ± 156.25	0.253	4.901 ± 0.0673	1.374
Flow Rate (1.0 ml/min)	0.8 ml/min	61123 ± 97.572	0.159	4.879 ± 0.00094	0.193
	1.2 ml/min	61903± 127.98	0.206	4.846± 0.0055	1.139

Table 16: Quantitative Analysis Data of Synthetic Mixture using HPLC

Synthetic Mixture (Tablet)	**Actual concentration (µg/ml)**		**Amount obtained (µg/ml)**		**GLM %Purity ± S.D. (n=5)**	**LIN %Purity ± S.D. (n=5)**
	Glimepiride	**Linagliptin**	**Glimepiride**	**Linagliptin**	99.16 ± 0.00385	99.20 ± 0.00480
	2	5	1.950 ± 0.0561	4.936 ± 0.0207		

RESULTS.

The technique demonstrated high linearity across the studied concentration range, with high correlation factors (r^2 = 0.9996 for GLM and 0.9993 for LIN) presented in figure 9, which provided a strong linear relationship between detector response and analyte concentration. The LOD and LOQ were 0.0875 µg/mL and 0.2651 µg/mL for Glimepiride encapsulated in table 14, and 0.0821 µg/mL and 0.2487 µg/mL for Linagliptin encapsulated in table 14, respectively, showing high sensitivity.

Accuracy was determined by recovery studies, giving 99.21–99.52% for Glimepiride results and 98.51–99.94% for Linagliptin results are presented in table 6, showing little interference from excipients. Precision testing, such as repeatability presented in table 11, intra-day presented in table 12, and inter-day presented in table 13 evaluation, illustrated relative standard deviation (%RSD) values much lower than the acceptance limit of 2%, guaranteeing method reproducibility. The technique was again confirmed by the analysis of synthetic mixture samples, wherein recovery percentages of 99.16% and 99.20% for Glimepiride and Linagliptin were found presented in table 6 and statistical recovery shown in table 10, proving it suitable for the analysis of real samples presented in table 16.

Moreover, studies of robustness demonstrated that small variations in mobile phase composition, flow rate, and wavelength detection did not have any significant effect on the performance of the method, attesting to the method's stability and performance under different conditions presented in table 15.

DISCUSSION.

A new and reliable HPLC method was established and validated for the co-estimation of Glimepiride (GLM) and Linagliptin (LIN) in bulk and synthetic mixtures.

The chromatographic conditions were maximized to obtain sharp, well-resolved peaks with low retention time. The chosen mobile phase ratio (Acetonitrile: Water, 70:30 v/v). provided good separation and result reproducibility. Glimepiride and Linagliptin were eluted at 4.884 min and 1.984 min depicted in figure 5, respectively, without interference from excipients, establishing method specificity.

NOVEL APPROACH OF THIS RESEARCH STUDY.

It’s much greener and simpler: We created a fast, eco-friendly testing method to measure two drugs (Glimepiride and Linagliptin) at the same time. Usually, scientists have to mix in extra chemicals like methanol or harsh acids (like orthophosphoric acid) to get clear results. Our method does the job perfectly without any of those extra additives.

It’s incredibly fast: By using a smart statistical testing layout (called Central Composite Design) to figure out how different settings affect each other, we fine-tuned the process. This allowed us to cut the traditional testing time down to just 10 minutes.

It’s highly sensitive and accurate: Even though it is faster and uses fewer chemicals, the method is a huge breakthrough in sensitivity. It can detect even tiny, trace amounts of the drugs or their impurities 0.0875 µg/mL and 0.0821 µg/mL. The final readouts are clean and sharp, making it highly reliable for testing low-strength medications without losing any accuracy.

CONCLUSION.

Experiments established that the HPLC analysis is capable of simultaneously measuring Glimepiride and Linagliptin with good accuracy in bulk drugs and synthetic mixtures. The method was found to be highly precise, accurate, sensitive, and reproducible, complying with the ICH guidelines for analytical validation. The short retention time, low solvent consumption, and fine resolution render this technique an inexpensive and efficient method for routine quality control analysis of Glimepiride and Linagliptin in drug formulations. Due to its sturdy

performance, the method can be easily used for pharmaceutical quality control and regulatory compliance in drug formulation analysis. Overall, the method is a merger of simplicity, accuracy, and precision and hence apt for use on a day-to-day basis.

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AUTHOR'S CONTRIBUTIONS
Patel Ayushi Shailesh bhai performed the experimental work and drafted the manuscript. Dr. Hiralben Mehta supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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

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REFERENCES.

1. National Center for Biotechnology Information. (2004). PubChem Compound Summary for CID 3476, Glimepiride. Retrieved October 12, 2024, Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Glimepiride
2. National Center for Biotechnology Information. (2004). PubChem Compound Summary for CID 10096344, Linagliptin. Retrieved October 12, 2024, Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Linagliptin
3. Marina Juliet A, Thanikachalam PV. Development and Validation of QBD-Assisted Using Central Composite Design RP-HPLC Method for Lobeglitazone Sulfate and Glimipiride in Bulk and Its Combined Dosage Form. Chromatographia. 2025 May;88(5):381-93.
4. Guedes, E. P., Hohl, A., De Melo, T. G., & Lauand, F. (2013). Linagliptin: Pharmacology, efficacy and safety in type 2 diabetes treatment. Diabetology & Metabolic Syndrome, 5, 1-7
5. Neumiller, J. J., & Setter, S. M. (2012). Review of linagliptin for the treatment of type 2 diabetes mellitus. Clinical Therapeutics, 34(5), 993-1005.
6. Ks. P., Dp, D., Sb, N., & Sanjaykumar, K. (2024). Development and validation of UV-spectroscopic method for simultaneous estimation of Glimepiride and Linagliptin in their synthetic mixture. International Journal of Pharmaceutical Research and Applications, 9(3), 2536-2545.
7. Falguniben, D., Patel, M., Marvaniya, V., & Prajapati, L. (2024). Development and validation of RP-HPLC method for simultaneous estimation of Glimepiride and Linagliptin from their synthetic mixture. International Journal of Novel Research and Development, 9(4), 787-802.
8. Godal, A., & Hinge, M. (2024). Reverse phase HPLC method development and validation for simultaneous estimation of Glimepiride and Linagliptin. International Journal of Emerging Technologies and Innovative Research, 118, a748-a761.
9. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*. 2008;76(5):965–977.
10. Ferreira SLC, Bruns RE, Ferreira HS, Matos GD, David JM, Brandão GC, et al. Box–Behnken design: An alternative for the optimization of analytical methods. *Anal Chim Acta*. 2007;597(2):179–186.
11. Beg S, Sharma G, Katare OP, Lohan S, Singh B. Development and optimization of pharmaceutical formulations using response surface methodology: A review. *Pharm Dev Technol*. 2015;20(3):273–286.
12. Prexa T, Shaileshbhai PA, Satishchandra MH. Spectroscopic Determination of Glimepiride and Linagliptin in Combined Synthetic Blends. Journal of Young Pharmacists. 2025 Jun 9;17(2):454-62.