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Original Article

A Systematic Design Of Experiment-Driven High Performance Thin Layer Chromatography Approach for the Quantification of Glimepiride and Linagliptin in Synthetic Blends

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ABSTRACT

Background: Type 2 Diabetes Mellitus (T2DM) often requires combination therapy for improvement of blood glucose regulation, where Linagliptin (a DPP-4 inhibitor) and Glimepiride (a sulfonylurea) act synergistically. Quality assurance of such combinations necessitates dependable on analytical methods. However, reports on their simultaneous estimation are limited. The present study focuses on developing and validating a precise, accurate, and robust HPTLC method for the quantification of Linagliptin and Glimepiride in bulk and synthetic mixtures using a Design of Experiments (DoE) approach. Hence the present manuscript describes the development and subsequent validation of a precise, accurate and robust HPTLC method for the simultaneous quantification of Glimepiride and Linagliptin in bulk as well as synthetic mixture using DoE approach. **Materials and Methods:** The HPTLC method was performed using silica gel 60 F_{254} pre-coated plates as the stationary phase, with a mobile phase consisting of a Methanol: Toluene: Ethyl Acetate: Chloroform [2:3:0.5:4.5 v/v/v/v] mixture and detection being carried out densitometrically at 259 nm. The validated method was found to be in conformance to ICH Q2 (R2) guideline and suitable for system suitability, linearity, accuracy, precision and robustness. However, keeping solvent front in view the robustness was studied by fractional factorial design (2^{4-1}), taking into account variables such as solvent front, wavelength, ethyl acetate volume, and chamber saturation time. **Results:** The current novel method is found to be linear (R^2>0.998), highly sensitive (Low LOD and LOQ concentrations), highly accurate (recovery is equivalent to 99-100%), highly precise (%RSD <2%), robust (against minor variations). **Conclusion:** The HPTLC method developed is simple, precise, accurate, and robust for the concurrent estimation of Glimepiride and Linagliptin in synthetic mixtures. Employing a fractional factorial design ensured the method's dependability under various conditions, making it ideal for routine pharmaceutical quality control.

Keywords: Fractional Factorial Design, Glimepiride, HPTLC, Linagliptin, Method Validation, Robustness.

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INTRODUCTION

Type 2 diabetes mellitus is referred as increasing blood sugar level which requires combination therapies for improvement of blood glucose regulations. where Linagliptin (a DPP-4 inhibitor) and Glimepiride (a sulfonylurea) act synergistically (Beckett, 2007; Indian Pharmacopoeia Commission, 2022; Mohan, 2019; Ojo, 2023; Guedes, 2013; Neumiller, 2012).

To ensure this drug combination is safe and effective, we need a dependable method to check its quality and stability. Although techniques like HPLC and spectroscopy have been used to test each drug separately, there isn't a well-established method for testing both at the same time in a lab-prepared mixture.

High-Performance Thin-Layer Chromatography (HPTLC) could be a great option because it's simple, fast, cost-effective, and allows testing of multiple samples at once (Neumiller, 2012; International Council for Harmonization, 2023; Ks, 2024; Falguniben, 2024).

Therefore, this study focuses on the development and validation of a simple, rapid, and accurate HPTLC method for the simultaneous determination of GLP and LNG in a synthetic mixture. Using Design of Experiments principles to test the robustness of High-Performance Thin-Layer Chromatography

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(HPTLC) techniques guarantees a methodical, scientific approach to method optimization. Finding important method variables and evaluating their effect on method performance within a specified design space are made easier with the aid of Design of Experiment (DoE). This tactic increases method reliability while reducing variability.

MATERIALS AND METHODS

Drug Components and Reagents

Glimepiride and linagliptin working standards were generously given as a free sample by Glenmark Pharmaceuticals Ltd. in Ankleshwar, India. The study's solvents and chemicals were all acquired from Merck Specialities Pvt. Ltd., in India.

Glimepiride 2 mg and Linagliptin 5 mg in combined tablet dosage form is approved by Central Drugs Standard Control Organisation (CDSCO) Indian regulatory authority. Hence, to ensure the accurate dose incorporated in tablet dosage form, this novel approach has been discovered.

HPTLC Systems

The chromatographic analysis was conducted using CAMAG instrumentation from Switzerland. The auto-sampler employed was the CAMAG-LINOMATE 5, equipped with a Hamilton syringe specifically designed for precision sampling. The Twin Trough chamber (20 × 20 cm) from CAMAG was utilized as the developing chamber, ensuring controlled and uniform plate development. The detection of analytes was performed using the CAMAG-TLC Scanner 4, which offers high sensitivity and accuracy. Data acquisition and analysis were carried out using Vision CATS software, version 13, which is optimized for advanced TLC method development and result interpretation.

Preparation of Standard Solution (Glimepiride 20 µg/mL, Linagliptin 50 µg/mL)

Transferred 1 mL of Glimepiride solution (200 µg/mL) and 1 mL of Linagliptin solution (500 µg/mL) were transferred into a 10 mL volumetric flask. The volume was made up to the mark with methanol to obtain a working standard solution containing 20 µg/mL of glimepiride and 50 µg/mL of linagliptin.

Preparation of Synthetic Mixture as per approved by Central drug standard control organisation

A synthetic mixture of Glimepiride and Linagliptin was prepared by accurately weighing 2 mg of Glimepiride and 5 mg of Linagliptin along with 77 mg of Microcrystalline Cellulose, 20 mg of Starch, 10 mg of Hydroxypropyl Cellulose, 4 mg of Talc, and 2 mg of Magnesium Stearate. All components were transferred to a clean, dry glass mortar and triturated uniformly using a pestle to obtain a homogeneous powder blend. The prepared mixture was employed for subsequent analytical evaluation and method validation.

Method Validation

System Suitability

System suitability testing proves the method's suitability. A standard solution of Glimepiride and Linagliptin was spotted five replicates at 2000 ng/band and 5000 ng/band Respectively. The retardation factor and peak area were measured using specified chromatographic conditions.

Specificity

The ability of an analytical technique to precisely quantify a target analyte in the presence of other elements that are probably present in the sample matrix is known as specificity, as the name suggests. The experiment utilized excipients such as cross carmellose sodium, talc, magnesium stearate, microcrystalline cellulose, starch, and hydroxypropyl cellulose to prepare the synthetic mixture. There are no interferences of any excipients used in preparation of Synthetic mixture (placebo).

Linearity and Range

At six concentration levels within the ranges of 2000-10000 ng/band (for Glimepiride) and 5000-25000 ng/band (for Linagliptin), the method's linearity was assessed through regression analysis. Peak area was plotted against concentration (n=5) to create the calibration curves. Results are shown in Table 5.

Precision

Precision was evaluated through repeatability, intra, and interday measurements. Repeatability involved six measurements of the same concentration Glimepiride (2000 ng/band) and Linagliptin (5000 ng/band) within a day, computing mean standard deviation and % RSD.

To determine intra-day precision, sample solutions of Glimepiride (2000, 4000, 6000 ng/band) and Linagliptin (5000, 10000, 15000 ng/band) were analysed at three levels concentrations of the calibration curve on the same day (n=3).

The inter-day precision was assessed by examining sample solutions of Glimepiride (2000, 4000, and 6000 ng/band) and Linagliptin (5000, 10000, and 15000 ng/band) at three concentration levels across three days (n=3). Mean values and relative standard deviation (% RSD) were calculated based on the peak areas obtained.

Accuracy

To determine the method's accuracy, the % recovery of Glimepiride and Linagliptin were calculated. Glimepiride and Linagliptin were added in known quantities at 80%, 100%, and 120% to a pre-quantified physical mixture. The solutions were spotted to the TLC plate, and development took place. The peak area was assessed, and the quantities of Glimepiride and Linagliptin were determined.

DL and QL

Detection and quantification were determined through the calibration curve method. The standard deviation of the y-intercepts of regression lines is used as the standard deviation. It was calculated as per ICH guidelines as described below:

$$(DL) = 3.3 \times \sigma/S$$

$$(QL) = 10 \times \sigma/S$$

"σ" stands for the standard deviation of regression y-intercepts, while "S" is the slope of a calibration curve.

Robustness Study

To evaluate the robustness of the developed HPTLC method, a Fractional Factorial Design (FFD) of type 2^{4-1} was employed. (**Software:** Design Expert version 13 was used for both experimental design and optimization analysis). This approach enabled the systematic assessment of four critical method parameters with a limited number of experiments. The selected factors were solvent front distance (A), detection wavelength (B), volume of ethyl acetate in the mobile phase (C), and chamber saturation time (D). Each parameter was varied at two levels high and low to simulate minor, deliberate changes from the optimized method conditions. All experiments were carried out in randomized order to minimize the impact of uncontrolled variables. The synthetic mixture of Glimepiride and Linagliptin was spotted onto HPTLC plates and analysed under the specified conditions for each experimental run. The Retention Factor (R_f) values for both drugs were recorded and assessed for variability to determine the method's robustness.

Analysis of Glimepiride and Linagliptin in Synthetic Mixture

A synthetic mixture equivalent to 2 mg of Glimepiride and 5 mg of Linagliptin (650 mg total) was transferred to a 100 mL volumetric flask, dissolved in methanol, and ultrasonicated for 20 min. The solution was filtered through a 0.45 µm Whatman filter paper and diluted to obtain 2 µg/mL of Glimepiride and 5 µg/mL of Linagliptin. Aliquots were applied to HPTLC plates, developed using the optimized mobile phase, and scanned at 259 nm. Quantification was performed using the respective calibration curves.

RESULTS

The developed method demonstrated excellent linearity over the tested concentration ranges of 2000-10000 ng/band for Glimepiride and 5000-250000 ng/band for Linagliptin, with correlation coefficients of 0.9981 and 0.9995, respectively. The Retention factor (R_f) values were found to be 0.655 for Glimepiride and 0.210 for Linagliptin.

The limits of detection were 229.129 ng/band for Glimepiride and 763.76 ng/band for Linagliptin, while the limits of quantification were 95.262 ng/band and 288.67 ng/band, respectively, indicating high sensitivity of the method.

Accuracy studies showed recovery values between 99.30% and 100.30%, whereas precision studies yielded %RSD values below 2%, confirming the reliability of the method. Robustness evaluation revealed that small variations in chromatographic conditions had no significant effect on retention factors, indicating the robustness of the method.

DISCUSSION

Type 2 diabetes mellitus is characterized by increased blood glucose levels and often requires combination therapy for effective glycemic control. Glimepiride and Linagliptin are commonly used for blood sugar regulation. A combined tablet dosage form containing Glimepiride 2 mg and Linagliptin 5 mg has been approved by the Central Drugs Standard Control Organisation (CDSCO). Therefore, ensuring accurate drug content in such formulations is essential, which led to the development of this method (Figure 1).

Among the different combinations evaluated, the mobile phase consisting of Methanol: Toluene: Ethyl Acetate: Chloroform (2:3:0.5:4.5, v/v/v/v) provided optimal separation (Figure 2). This composition produced well-defined bands with consistent R_f values and minimal tailing, and was selected for further HPTLC analysis (Table 1).

System suitability was evaluated by performing five replicate analyses at concentrations of 2000 ng/band for Glimepiride and 5000 ng/band for Linagliptin under fixed chromatographic conditions. The method demonstrated good specificity, with no interference observed at the respective R_f values of the analytes, as shown in Figures 2 (A, B). The calibration curves showed strong linear relationships, with correlation coefficients (R^2) of

Table 1: Experimental factors and levels used in FFD (2^{4-1}).

Sl. No.	Factors	High level	Low level
1.	Wavelength (nm)	258	260
2.	Volume of Ethyl acetate (mL)	0.4	0.6
3.	Solvent front (mm)	81	79
4.	Chamber saturation time (min)	18	22

0.9981 for Glimepiride and 0.9995 for Linagliptin, as presented in Figures 3, 4, and Table 2.

Precision studies, including repeatability, intraday, and interday assessments, showed %RSD values below 2% for all standards. The results of intraday precision are shown in Table 3, while interday precision results are presented in Table 4. Accuracy studies demonstrated recovery values within the range of 98-102%, with %RSD values below 2%, complying with ICH guidelines (Table 5). The method was also capable of detecting and quantifying small amounts of both drugs with high precision (Table 6).

Robustness studies were carried out using Design Expert version 13, and the factorial model for the R_f values of both drugs is presented in Table 7. Model validation using ANOVA is shown in Table 8. The adequate precision values were above 4, indicating a satisfactory signal-to-noise ratio. The *p*-values obtained were greater than 0.05, indicating that the models were statistically non-significant. This confirms that small deliberate variations in experimental conditions do not significantly affect the method performance.

Perturbation plots were used to evaluate the influence of solvent front (A), wavelength (B), volume of ethyl acetate (C), and chamber saturation time (D) on the R_f values. For Glimepiride, all factors showed minimal influence, with slight downward trends observed for A and B, while C and D remained nearly constant, as shown in Figure 4. For Linagliptin, factors A, C, and D showed slight decreasing trends, whereas factor B remained almost unchanged, as shown in Figure 5. The absence of sharp variations confirmed that these parameters do not significantly affect the chromatographic response.

Three-dimensional response surface plots further supported these findings. For Glimepiride, interactions between solvent front and wavelength (Figure 6), solvent front and ethyl acetate volume (Figure 7), and solvent front and chamber saturation time (Figure 8) showed nearly flat or slightly varying surfaces. For Linagliptin,

Table 2: Method Linearity Parameters.

Conc. (ng/band)	Glimepiride		Conc. (ng/band)	Linagliptin	
	Mean Peak Area	%RSD		Mean Peak Area	%RSD
2000	0.00115	1.332	5000	0.00123	0.468
4000	0.00157	0.733	10000	0.00227	0.918
6000	0.00202	0.990	15000	0.00324	0.993
8000	0.00253	0.455	20000	0.00421	0.725
10000	0.00304	1.24	25000	0.00521	0.880
Regression Equation	y = 2E-07x + 0.0006		Regression Equation	y = 2E-07x + 0.0003	
R^2	0.9981		R^2	0.9995	
Slope	0.0000002		Slope	0.0000002	
Intercept	0.0006		Intercept	0.0003	
Linearity Range (ng/band)	2000-10000		Linearity Range (ng/band)	5000-25000	

*n=3 replicates.

Table 3: Intraday Precision of Glimepiride and Linagliptin.

Conc. (ng/band)	Intraday Precision Peak Area			Mean Peak Area	SD	%RSD
	1 hr.	2 hr.	3 hr.			
Glimepiride						
2000	0.00114	0.00116	0.00115	0.00115	0.0000100	0.869
4000	0.00156	0.00154	0.00158	0.00156	0.0000221	1.282
6000	0.00247	0.00244	0.00245	0.00245	0.0000153	0.622
Linagliptin						
5000	0.00139	0.00140	0.00138	0.00139	0.0000103	0.739
10000	0.00204	0.00208	0.00210	0.00207	0.0000306	1.473
15000	0.00383	0.00386	0.00385	0.00385	0.0000252	0.397

*n=3.

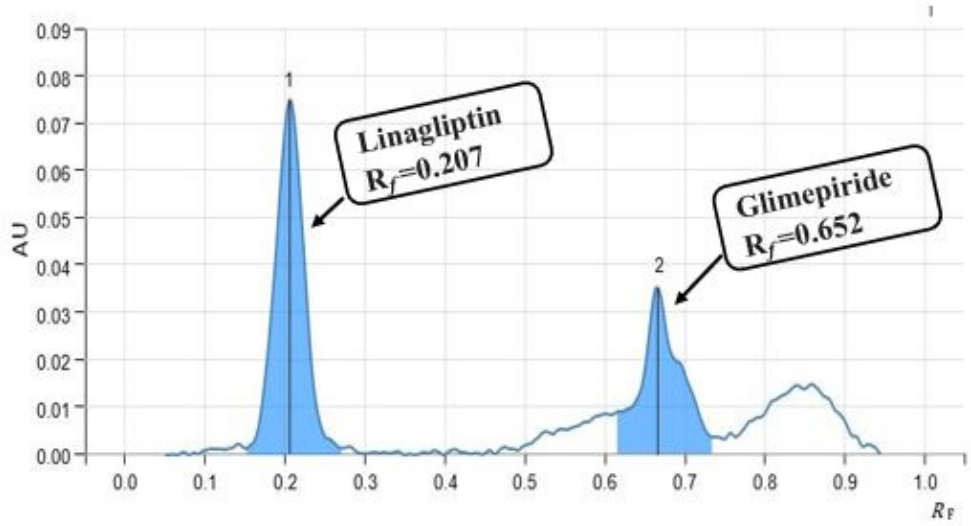

Figure 1: HPTLC Chromatogram of Synthetic Mixture of Glimepiride and Linagliptin.

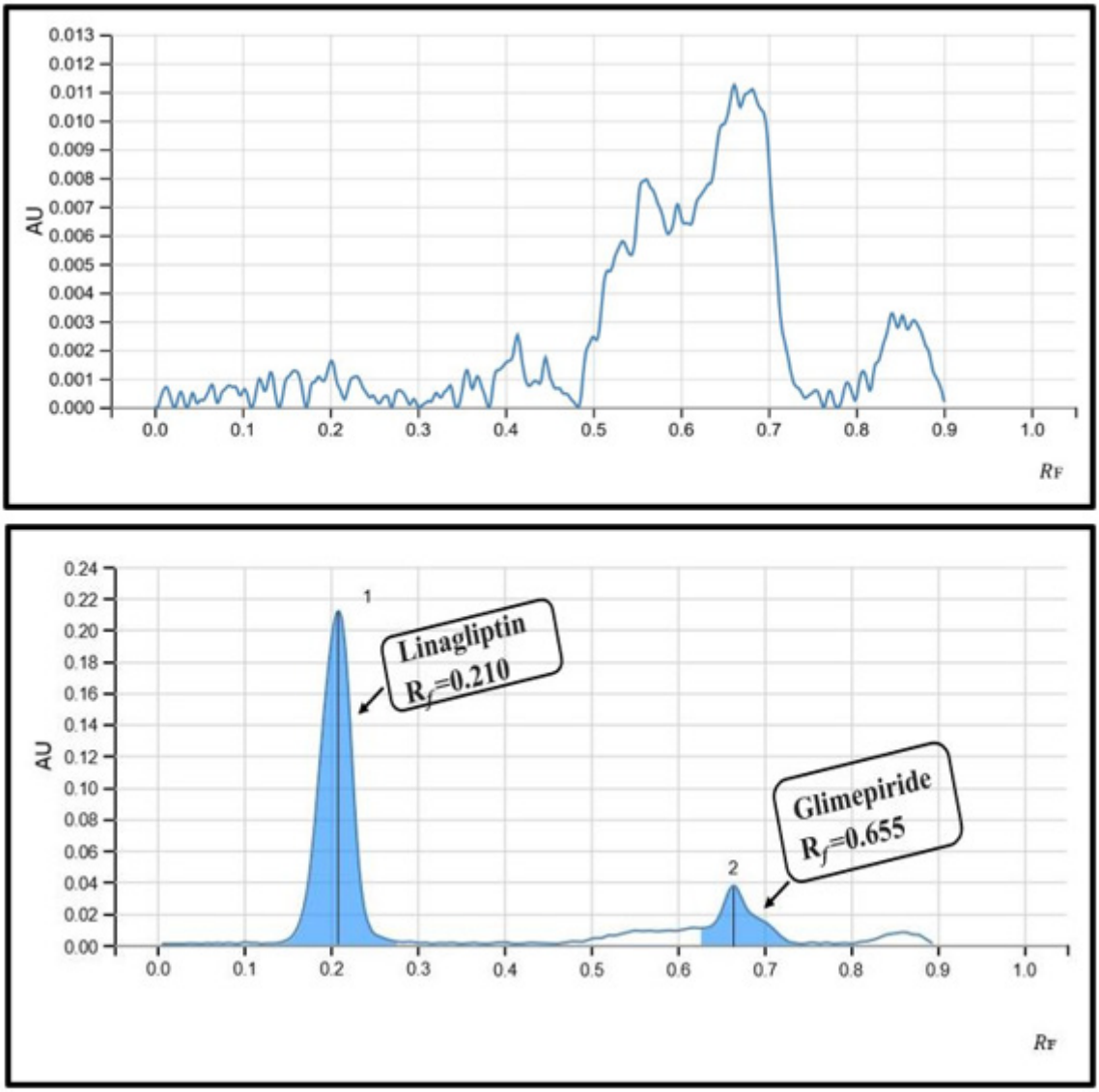

Figure 2: Chromatogram of Synthetic mixture without API (Placebo) (A) and Synthetic mixture with API (B).

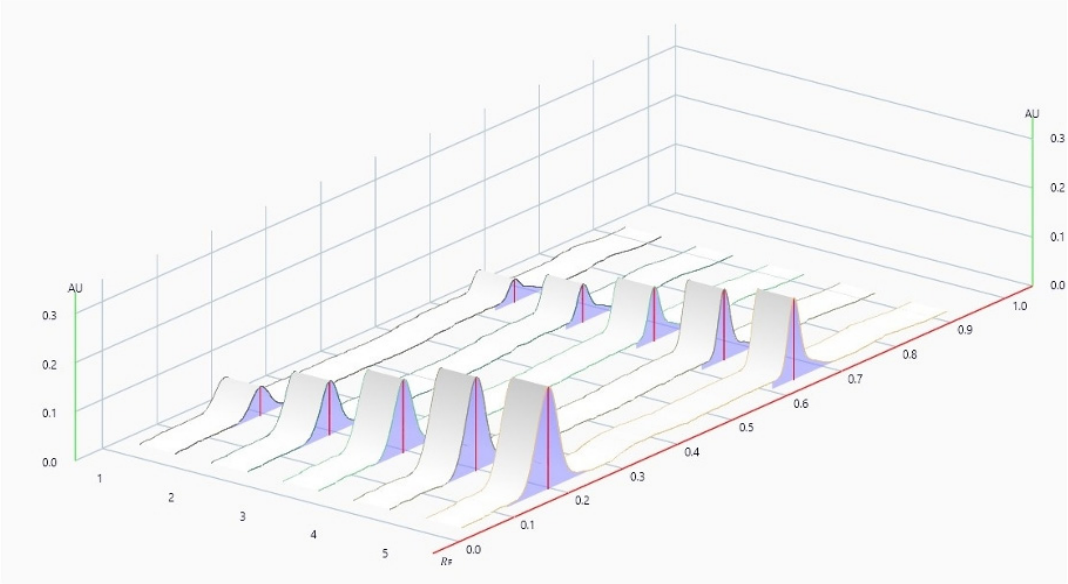

Figure 3: 3D Densitogram of Glimepiride and Linagliptin in Synthetic Mixture.

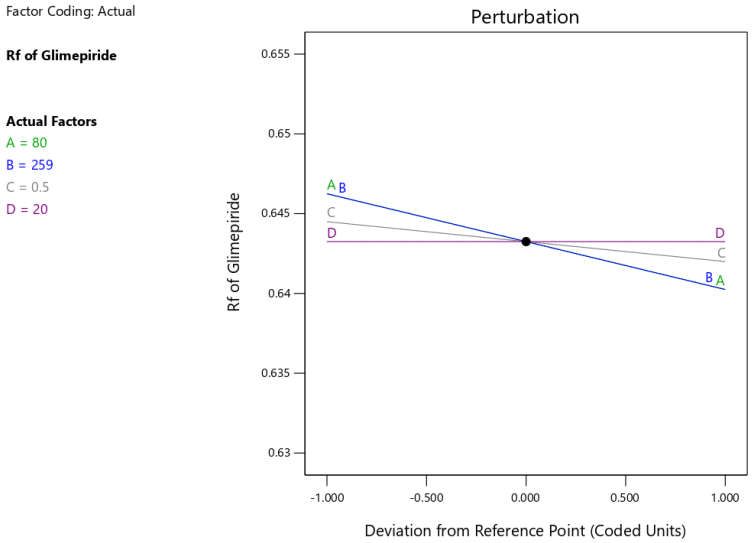

Figure 4: Perturbation Plot Showing Effects of Various Factor on Response of Glimepiride.

Table 4: Interday Precision of Glimepiride and Linagliptin.

Conc. (ng/band)	Interday Precision			Mean Peak Area	SD	%RSD
	Peak Area					
	Day 1	Day 2	Day 3			
Glimepiride						
2000	0.00109	0.00111	0.00112	0.00111	0.0000153	1.380
4000	0.00182	0.00186	0.00184	0.00184	0.0000200	1.087
6000	0.00252	0.00253	0.00256	0.00254	0.0000208	0.821
Linagliptin						
5000	0.00146	0.00140	0.00143	0.00144	0.0000208	1.449
10000	0.00211	0.00214	0.00214	0.00213	0.0000173	0.813
15000	0.00399	0.00394	0.00392	0.00395	0.0000361	0.913

*n=3.

Table 5: Accuracy Study Data of Glimepiride and Linagliptin.

Standard	% Spiked	Amount Present (ng/band)	Addition Amount (ng/band)	Quantity of sample found (ng/band) Mean	Mean % Recovery	% RSD
Glimepiride	80 %	2000	1600	3576	99.33	0.65
	100 %	2000	2000	3973	99.32	0.49
	120 %	2000	2400	4369	99.90	0.38
Linagliptin	80 %	5000	4000	8992	99.91	0.11
	100 %	5000	5000	9998	99.98	0.19
	120 %	5000	6000	10996	99.96	0.59

*n=3, % RSD < 2.

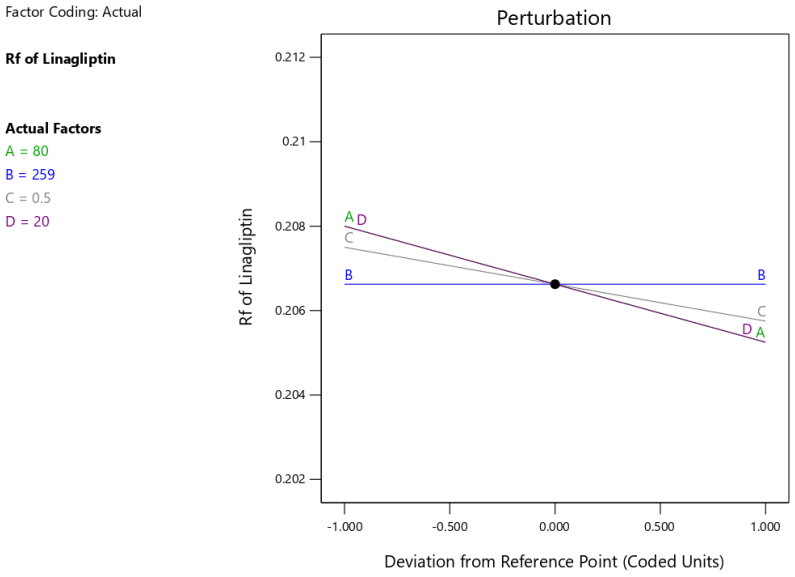

Figure 5: Perturbation Plot Showing Effects of Various Factor on Response of Linagliptin.

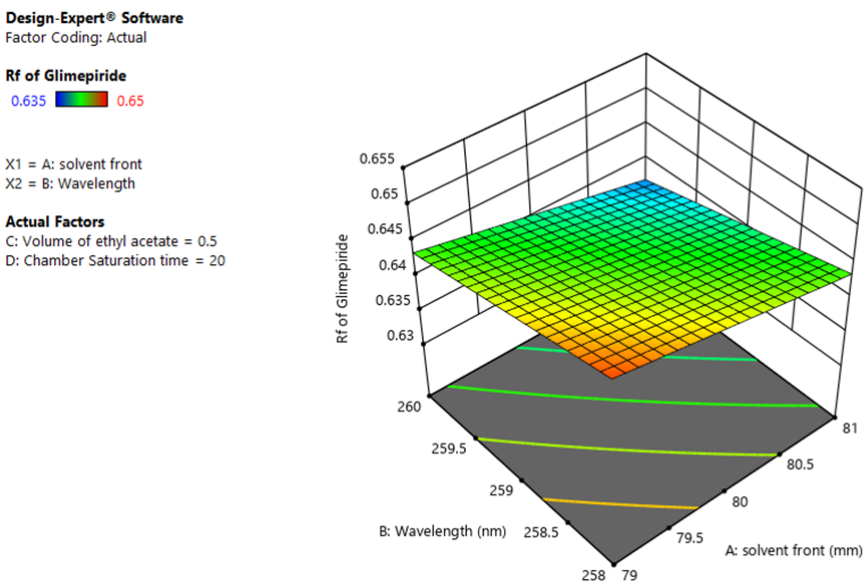

Figure 6: 3D Response Surface Plot for the Retention Factor of Glimepiride (A and B).

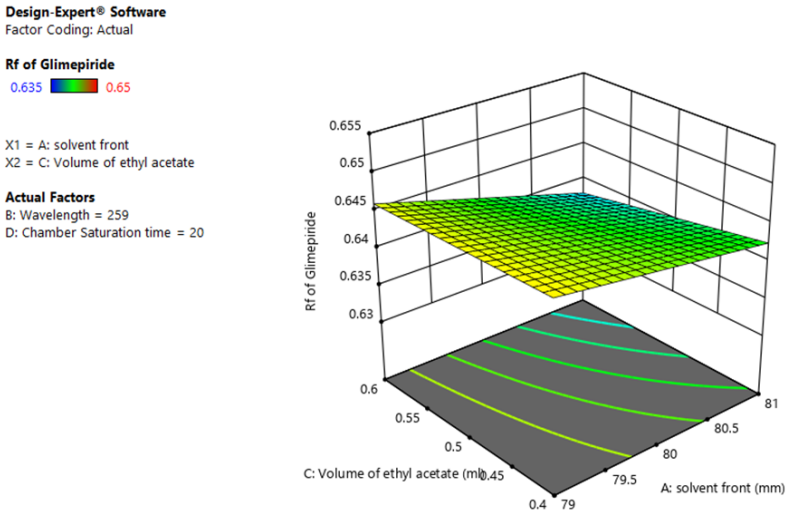

Figure 7: 3D Response Surface Plot for the Retention Factor of Glimepiride (A and C).

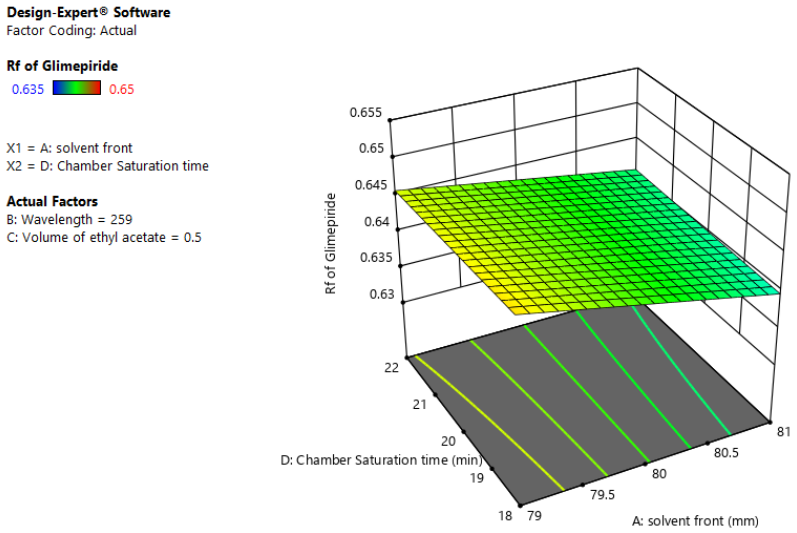

Figure 8: 3D Response Surface Plot for the Retention Factor of Glimepiride (A and D).

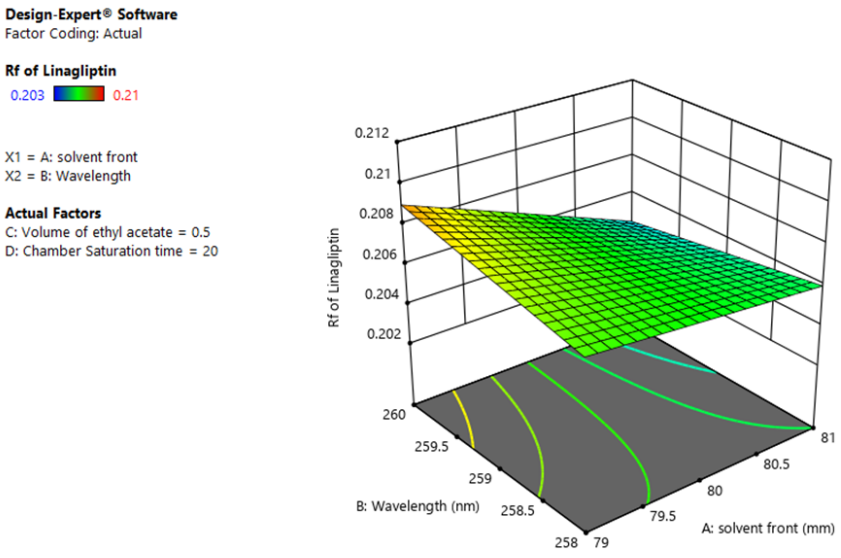

Figure 9: 3D Response Surface Plot for the Retention Factor of Linagliptin (A and B).

Table 6: DL and QL Values of Glimepiride and Linagliptin.

Drug	Detection Limit (DL) (ng/band)	Quantitation Limit (QL) (ng/band)
Glimepiride	229.129	763.76
Linagliptin	95.262	288.67

Table 7: Fractional Factorial Design Obtained Responses.

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2
Run	A: solvent front	B: Wavelength	C: Volume of ethyl acetate	D: Chamber Saturation time	R_f of Linagliptin	R_f of Glimepiride
	mm	nm	mL	min	-	-
1	81	260	0.6	22	0.203	0.635
2	79	258	0.6	22	0.204	0.648
3	79	260	0.4	22	0.209	0.643
4	79	258	0.4	18	0.210	0.650
5	81	258	0.6	18	0.207	0.641
6	81	258	0.4	22	0.205	0.646
7	81	260	0.4	18	0.206	0.639
8	79	260	0.6	18	0.209	0.644

Table 8 (A): ANOVA for Responses of R_f of Glimepiride.

R_f of Glimepiride						
Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0002	6	0.0000	55.67	0.1022	not significant
A-solvent front	0.0001	1	0.0001	144.00	0.0529	
B-Wavelength	0.0001	1	0.0001	144.00	0.0529	
C-Volume of ethyl acetate	0.0000	1	0.0000	25.00	0.1257	
AB	5.000E-07	1	5.000E-07	1.00	0.5000	
AC	8.000E-06	1	8.000E-06	16.00	0.1560	
AD	2.000E-06	1	2.000E-06	4.00	0.2952	
Residual	5.000E-07	1	5.000E-07			
Cor Total	0.0002	7				

Table 8 (B): ANOVA for Responses of R_f of Linagliptin.

R_f of Linagliptin						
Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.0000	6	7.625E-06	61.00	0.0977	not significant
A-solvent front	0.0000	1	0.0000	121.00	0.0577	
B-Wavelength	1.250E-07	1	1.250E-07	1.0000	0.5000	
C-Volume of ethyl acetate	6.125E-06	1	6.125E-06	49.00	0.0903	
D-Chamber Saturation time	0.0000	1	0.0000	121.00	0.0577	
AB	6.125E-06	1	6.125E-06	49.00	0.0903	
AC	3.125E-06	1	3.125E-06	25.00	0.1257	
Residual	1.250E-07	1	1.250E-07			
Cor Total	0.0000	7				

Table 9: Quantification of Synthetic Mixture.

Component	Expected amount (mg)	Concentration (ng/ band)	Found amount(mg)	% Assay±S.D. (*n*=5)
Glimepiride	178.57	17546	175.46	98.26%±0.20
Linagliptin	446.43	44551	445.51	99.79%±0.18

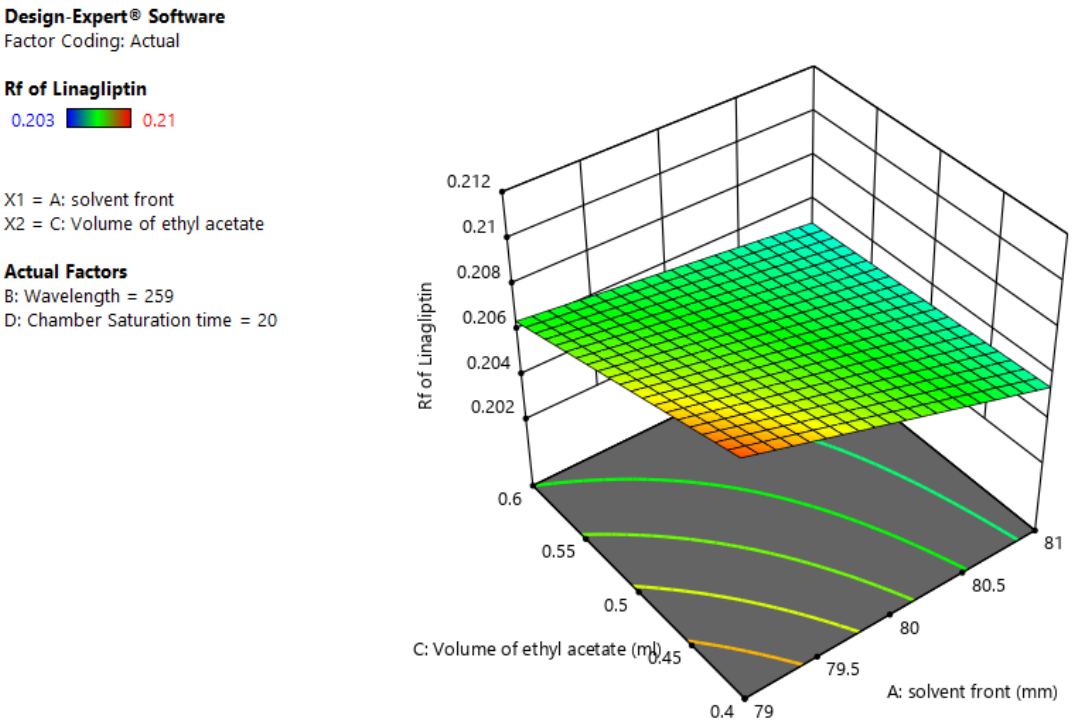

Figure 10: 3D Response Surface Plot for the Retention Factor of Linagliptin (A and C).

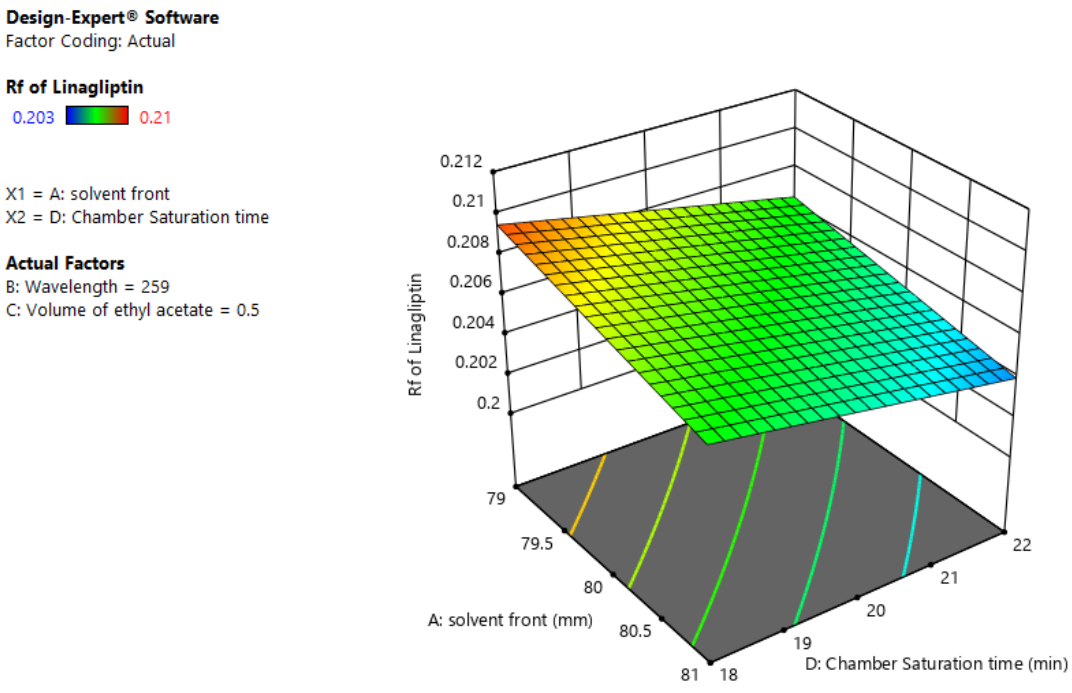

Figure 11: 3D Response Surface Plot for the Retention Factor of Linagliptin (A and D).

a slight decrease in R_f was observed with increasing wavelength (Figure 9), while interactions with ethyl acetate volume (Figure 10) and chamber saturation time (Figure 11) remained nearly uniform. These results indicate that the method is stable and robust, with minimal influence from the studied variables.

The quantification of the synthetic mixture confirmed the applicability of the developed method, as presented in Table 9.

CONCLUSION

The current novel method is found to be linear (R^2>0.998), highly sensitive (Low LOD and LOQ concentrations), highly accurate (recovery is equivalent to 99-100%), highly precise (%RSD <2%), robust (against minor variations). Its successful application to synthetic mixtures further highlights its practical utility, making it an ideal tool for pharmaceutical quality control and regulatory compliance.

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ABBREVIATIONS

ANOVA: Analysis of Variance; **CDSCO:** Central Drugs Standard Control Organisation; **DL:** Detection Limit; **DoE:** Design of Experiments; **DPP-4:** Dipeptidyl Peptidase-4; **FFD:** Fractional Factorial Design; **HPLC:** High-Performance Liquid Chromatography; **HPTLC:** High-Performance Thin-Layer Chromatography; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **QL:** Quantitation Limit; **RSD:** Relative Standard Deviation; **R_f:** Retardation Factor; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **T2DM:** Type 2 Diabetes Mellitus; **TLC:** Thin-Layer Chromatography; **UV:** Ultraviolet.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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