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IMPLEMENTATION OF ANALYTICAL QBD IN STABILITY-INDICATING RP-UPLC METHOD DEVELOPMENT AND VALIDATION FOR COMBINED QUANTIFICATION OF VILDAGLIPTIN AND PIOGLITAZONE

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ABSTRACT
Concurrent detection of Vildagliptin and Pioglitazone in pharmaceutical formulations is the primary goal of this work, which aims to establish an RP-UPLC technique. An improved chromatographic environment was the goal of the methodology's Central Composite Design (CCD) phase. Three independent variables—temperature, mobile phase composition, and flow rate—were included in the design that was implemented using Design-Expert® software. With a UV detector set to 212 nm, the column temperature was maintained at 30°C, and a flow rate of 0.31 mL/min, the RP-UPLC analysis was carried out. For Pioglitazone and Vildagliptin, the approach was validated by analysing linearity, precision (%RSD), recovery rates, and forced degradation trials. In the advanced RP-UPLC method, Vildagliptin (12.5-75 µg/mL) and Pioglitazone (3.75-22.5 µg/mL) demonstrated strong linearity. Vildagliptin had a Limits of Detection of 0.44 µg/mL and pioglitazone had a quantification threshold of 1.32 µg/mL, while pioglitazone had a threshold of 4.02 µg/mL. For both Vildagliptin and Pioglitazone, the recovery rates ranged from 99.08% to 100.98%, and the methodology's Relative Standard Deviation (RSD) was less than 2% in terms of accuracy. Both chemicals were susceptible to oxidative and photolytic reactions, according to studies on synthetic deterioration. Using the validated RP-UPLC method, which was designed in accordance with Quality by Design (QbD) principles, the pharmaceutical formulations including Vildagliptin and Pioglitazone were simultaneously quantified. Cases of induced degradation proved the stability, sensitivity, and extraordinary linearity of the approach.

KEYWORDS: Central Composite Design (CCD), Vildagliptin, Pioglitazone, AQbD and RP-UPLC.

INTRODUCTION
Vildagliptin (VGT) is a targeted pyrrolidine-2-carbonitrile that functions as an inhibitor of dipeptidyl peptidase-4 (CD26) [1]. Figure 1A illustrates the compound, which has the molecular formula C17H25N3O2. This aids individuals with diabetes in effectively managing their condition. VGT is an antidiabetic drug that helps control type 1 diabetes by blocking the enzyme dipeptidyl peptidase-4 [2]. Because dipeptidyl peptidase-4 is unable to render GLP-1 inactive, beta cells are able to release more insulin when administered GLP-1[3]. Some research suggests that dipeptidyl peptidase-4 may degrade GIP and GLP-1 1,2 to help control blood sugar levels. 23% of the oral dose of vildagliptin is eliminated unaltered in the urine, with the remaining 85% being eliminated by renal excretion and the remaining 70% being metabolized by hydrolysis [4]. The drug's pharmacokinetics are unaffected by food consumption [5]. The main P450 enzymes are neither induced nor inhibited by it. Additionally, it does not exhibit pharmacological interactions with widely used medications, including amlodipine, ramipril valsartan, digoxin, simvastatin, metformin, glyburide, pioglitazone, warfarin, and ramipril [6] [7]. Age, gender, BMI, and race had no effect on vildagliptin's pharmacokinetics [8].Medication classified under thiazolidinediones Pioglitazone hydrochloride (PGT), [9-13] characterized by the molecular formula C19H21ClN2O3S (Figure 1B), functions as a hypo glycaemic agent. role in managing hyper glycemia and diabetes. While PPAR-α is mostly unaffected, the main target of stimulation is the nuclear receptor peroxisome proliferator activated receptor-gamma (PPAR-γ). By controlling PGT transcription, it regulates insulin-sensitive gene expression in muscle, adipose tissue, and the liver, all of which play an essential role in controlling glucose and lipid metabolism. When peripheral tissues, including the liver, develop insulin resistance, insulin levels, glucose, and glycosylated haemoglobin all drop, the cost of insulin-dependent glucose goes up, and glucose outflow from the liver goes down 3,4.

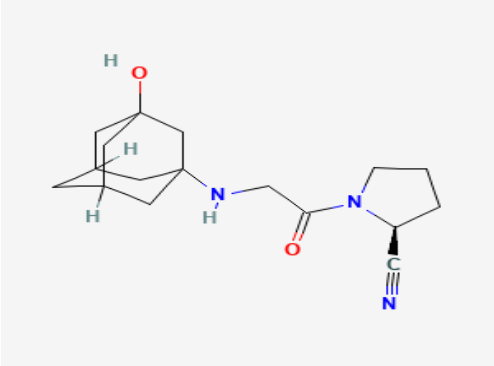


Fig 1 (A): Vildagliptin Structure

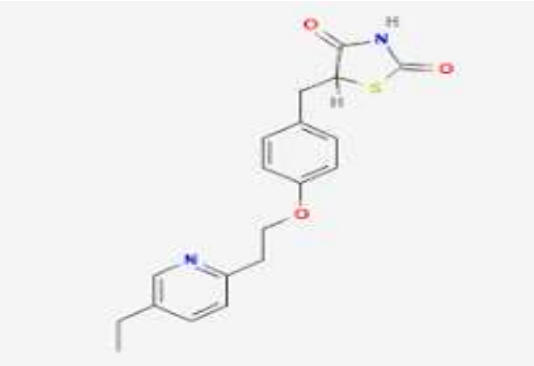


Fig 1 (B): Pioglitazone Structure

Since present methods can only analyse and detect VGT and PGT independently, that includes PGT in UV [14], HPLC [15], FTIR [16], HPTLC [17], and VGT done in individually or other combinations, includes UV [18-19], HPLC [20-21], HPTLC [22], LC-MS [23] and plasma [24]. It is crucial to develop a new approach that can overcome the limits of current technology. A common method for drug measurement is high-performance liquid chromatography (HPLC) [25-27], it exhibits several limitations relative to more contemporary techniques like RP-UPLC, including reduced throughput, diminished sensitivity, and extended run times. A greater volume of mobile phase is required for HPLC, rendering it less environmentally sustainable and ultimately more costly. LC-MS is an exceptionally sensitive technology capable of detecting chemicals at remarkably low concentrations. Nonetheless, it necessitates specialized apparatus and incurs a substantial cost. Routine quality checks across the pharmaceutical business are sometimes impractical due to its complexity and substantial expenses. Conversely, RP-UPLC offers several advantages, including enhanced sensitivity, expedited analysis, and superior resolution, all of which are crucial for pharmaceutical formulations as they ensure precise and efficient measurement. Furthermore, RP-UPLC is ideal for regular analysis due to its cost-effectiveness, reduced sample requirements, and rapid results [29]. This research seeks to tackle these challenges and offer a reliable, efficient, and economical approach for quantifying VGT and PGT. It guarantees dependable stability testing and quality control in pharmaceutical products by optimizing an RP-UPLC method through Analytical Quality by Design (AQbD) principles [29-37].

2. MATERIALS AND METHODS

2.1 Materials

Zydus Cadila Health Care Ltd. of Secunderabad supplied medication samples (VGT and Vildagliptin) with a purity level of 99.9%. All trials utilized Vylda P 15 mg/50 mg Tablets in a sustained-release formulation. Components: distilled water, methanol, acetonitrile, triethylamine. Rankem Chemicals sources all of its chemicals and solvents from the Indian state of Haryana. Merck Millipore, an Indian enterprise, acquired Millipore Direct-Q®3 UV water purification apparatus due to the significant demand for ultrapure water.

2.2 Equipments

The investigation used a binary pump system, a C18 analytical column (at room temperature for 10 minutes, with a particle size of 5 µm, an internal diameter of 4.6×250 mm, and a flow rate of 1.00 ml/min, utilizing the WATERS Acquity UPLC System. An autosampler and a TUV detector linked to Empower 3. The PG Instruments T60 UV-Visible Spectrophotometer, designed for this study, had 2 mm and 10 mm bandwidths, respectively, carefully selected quartz cells, and UV Win 6 software. Additional equipment utilized included a pH meter, an ultrasonicator, an LG refrigerator, BVK Enterprises of India, and a Millipore BM2EA9672R.

2.3 Composition of solutions:

2.3.1 Preparation of Buffer: (monobasic sodium phosphate)

12.0 grams of monobasic sodium phosphate were incorporated into 900 millilitres of water. Adjust the volume to 1000 mL with water, then utilize phosphoric acid or 1 M hydrochloric acid to achieve a pH of 4.4 ± 0.1.

2.3.2 Mobile phase:

The mobile phase calls for 69.8 volumes of buffer and is made up of 30.2 volumes of acetonitrile, both of which must be filtered prior to use. The solubility of the medications guided our selection of diluent, specifically a 50:50 mixture of acetonitrile and water.

2.3.3 Preparation of Stock solutions: Volumetric flasks with a capacity of 50 ml were utilized to transfer 25 mg of VGT and 7.5 mg of PGT, respectively. Subsequent to the addition of three quarters of the diluents to the flask, the mixture underwent sonication for a duration of ten minutes. The initial standard stock solution was housed in a diluted flask. in combination with VGT at a dose of 500 µg/mL and PGT at 150 µg/mL.

To make the working standard solutions (100% solution), 15µg/ml of PGT and 50µg/ml of VGT were achieved by pipetting 1 ml from the stock solution into a 10-milliliter volumetric flask and diluting it.

2.3.4 Preparation of Sample stock solutions: We conducted an analysis by taking five tablets and calculating the average of their measurements to determine the quantities of VGT (300 mg) and PGT (100 mg) present in a single tablet. The 25-minute sonication operation was followed by the transfer of the liquid to a 100 mL volumetric flask. The average weight of the mixture was 482.6 milligrams. Then, the remaining volume was diluted with a diluent, and the combination was filtered using HPLC filters. Upon comparison, the concentrations of VGT were determined to be 500µg/ml, while PGT was discovered to be 150µg/ml.

2.3.5 Preparation of Sample working solutions (100% solution): A diluent was added to a 10-milliliter volumetric flask, and then one millilitre of stock solution was added. The concentration of PGT was measured at 15 µg/ml, while VGT was recorded at 50 µg/ml 8.

3. METHOD OPTIMIZATION THROUGH EXPERIMENTAL DESIGN

Utilizing Stat-expert®, a tool developed by Stat-Ease Inc. (Version 11.1.0.1, USA), we revised the chromatographic conditions of the algorithm. This program operates through the application of a central composite design (CCD) [28] at five distinct levels (− α, − 1, 0, + 1, and + α), which is comprised of three components. The pilot experiments indicated that acetonitrile and 0.1N buffer were selected as the mobile phase (X1), along with temperature (X3) and flow rate (X1). One resolution factor (Y1), two theoretical plate numbers (Y2 and Y3), one theoretical plate number (Y3) for PGT, one tailing factor (Y4) for VGT, and one tailing factor (Y5) for PGT were selected as dependent variables. The Design Expert® software, utilizing the primary composite configuration, recommended conducting twenty distinct tests. Each experiment utilized 50 µg/mL of VGT and 15 µg/mL of PGT. The measurements of the essential elements within the composite design:

Table 1: A set of independent factors:

'Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	FR	ml/min	Numeric	Continuous	0.2495	0.3505	-1 ↔ 0.27	+1 ↔ 0.33	0.3000	0.0254
B	MP	%	Numeric	Continuous	21.59	38.41	-1 ↔ 25.00	+1 ↔ 35.00	30.00	4.24
C	Temp	0 C	Numeric	Continuous	24.95	35.05	-1 ↔ 27.00	+1 ↔ 33.00	30.00	2.54'

'Y1: Resolution
Y2: Theoretical plates of VGT,
Y3: Theoretical plates of PGT,
Y4: Tailing factor of VGT,
Y5: Tailing factor of PGT'

4.Validation of Method [38-40]

In order to ensure that the novel RP-UPLC method meets the requirements set out by ICH Q2 (R1), its accuracy, precision, linearity, and low limit of detection (LOD) are validated. Six standard injections of 15 µg/mL PGT and 50 µg/mL VGT are given as part of the system suitability test. Validation criteria include theoretical plates, resolution, peak area, retention time percent RSD, and the procedure itself. Using working standard solutions of PGT (3.75-22.5 µg/mL) and VGT (12.5-75 µg/mL), a calibration curve was created to assess the linearity of the method by graphing peak area against concentration. To find the limits of detection (LOD) as 3.3 × σ/S and the limits of quantification (LOQ) as 10 × σ/S, the slope (S) and standard deviation (Q) of the calibration curve were used, respectively. To ensure the procedure's accuracy, we examined both its internal and external deviations. To ensure consistency, PGT and VGT were evaluated on different days at concentrations of 7.5, 15, and 22.5 µg/mL. They underwent testing for accuracy on different days at concentrations of 25, 50, and 75 µg/mL. We evaluated their accuracy by combining solution samples containing 50, 100, and 150 percent PGT and VGT with previously analyzed drug samples. Through a comparative analysis of the chromatograms from the control, placebo, and PGT/VGT standard solutions, we confirmed the validity of the method. The experimenters systematically modified the column temperature (± 3 °C), methanol percentage (± 5% in the mobile phase), and flow rate (± 0.3 mL) to evaluate longevity 9.

4.1 Consistency of the sample solution

After leaving the PGT and VGT solutions at room temperature for one day, the samples were moved to a volumetric flask to ensure their stability. Using the initial data as a benchmark, we compared the computed PGT peak areas and retention times to those of the following day's PGT.

4.2 Investigations on forced deterioration [41-50]

We conducted tests on PGT and VGT across different stress levels to determine their forced breakdown rate. Stress conditions applied to the PGT and VGT solutions involve heating them to 60 °C for a duration of 30 minutes in a 2N HCl solution. The evaluation of the PGT and VGT solutions involved photolysis through seven days of UV exposure, dry heat, neutral, water, acid, and alkaline treatments, along with a series of degradation tests. After exposure, the concentrations of PGT and VGT were modified to 15 °g/mL and 50 µg/mL, respectively. I introduced five microlitres of each solution into the instrument to assess the stability of the sample, subsequently acquiring chromatograms.

4.3 Evaluation using statistical methods

Following the calculation of the mean, we proceeded to adjust the figures by either adding or subtracting the standard deviation to arrive at the final values. A number of statistical measures, including the mean, standard deviation, slope, and standard deviation %, were calculated using Excel. Using analysis of variance (ANOVA), we looked for statistically significant correlations between the model and its parameters. All terms and the model were deemed significant when the p-value dropped below 0.05.

5 RESULTS

5.1 Studies on the development of methods

The exploration of many solvents, flow modes, columns, and temperatures resulted in the advancement of liquid chromatography (LC) technology for chemical separation. A diverse array of solvents was employed, including water, acetonitrile, methanol, ethanol, and a pH-controlled phosphate buffer. A combination of acetonitrile and 0.1 N phosphate buffer was found to be the most effective mobile phase for the separation of PGT and VGT in the initial research. This stage significantly surpasses others regarding peak performance, retention duration, and tailing characteristics.

5.2 Method optimisation using experimental design

To examine the impact of three independent variables on seven dependent variables, this research utilised a central composite design consisting of twenty trials. X1, representing the inflow rate, is one of three independent variables in the model; X2, indicating the mobile phase composition; and X3, representing the temperature. The dependent variables in this study are Y1 (solution factor), Y2 (theoretical plate counts), Y3, and Y4 and Y5 (tailing factors). This study examined three independent variables and their effects on five dependent variables across twenty trials utilizing a central composite design (table 2).

5.3 Respondents' model-fitting

The Design-Expert tool analysed the data to identify the ideal mathematical model by comparing the results of all twenty iterations. Table 3 presents standard deviations, correlation coefficients, predicted residual sums of squares (PRESS), model projected and adjusted R^2 values, and coefficients of variation (CVs). Ratios of adjusted and anticipated R2 values, standard deviation, correlation, coefficient of variation, and prediction sum of squares were among the numerous criteria employed to choose the models. Each response was meticulously scrutinized. The quadratic model was the optimal match for all seven responses (Y1-Y5).

Table 2: Optimal RP-UPLC method for VGT and PGT, including central composite design and observed values:

		'Factor 1	**Factor 2**	**Response 1**	**Response 2**	**Response 3**	**Response 4**	**Response 5**
Std	Run	A:FR	B:MP(Organic phase)	RS	NTPS1	NTPS2	TF1	TF2
		ml/min	%	min	min	num	num	num
2	1	0.33	25	1.34	7680	3265	1.96	2.23
3	2	0.27	35	2.64	8355	3820	1.25	1.24
18	3	0.3	30	1.8	8026	3533	1.58	1.56
13	4	0.3	30	1.8	8021	3529	1.59	1.57
11	5	0.3	21.591	1.22	7568	2989.7	2.45	2.47
19	6	0.3	30	1.8	8025	3529	1.58	1.56
15	7	0.3	30	1.8	8029	3555	1.57	1.54
20	8	0.3	30	1.8	8058	3535	1.57	1.57
16	9	0.3	30	1.8	8068	3525	1.56	1.55
4	10	0.33	35	2.7	8365	3830	1.24	1.23
8	11	0.33	35	2.65	8359	3850	1.23	1.23
14	12	0.3	30	1.83	8065	3560	1.57	1.56
17	13	0.3	30	1.84	8021	3535	1.56	1.56
9	14	0.249546	30	1.84	8026	3560	1.57	1.57
12	15	0.3	38.409	3.6	8520	3980	1.14	1.11
7	16	0.27	35	2.64	8360	3845	1.23	1.22
10	17	0.350454	30	1.8	8056	3525	1.57	1.57
1	18	0.27	25	1.34	7695	3270	1.97	2.23
5	19	0.27	25	1.32	7685	3265	1.99	2.23
6	20	0.33	25	1.3	7705	3260	1.97	2.24'

5.3.1 A number of external variables influence the resolution factor (Y1):

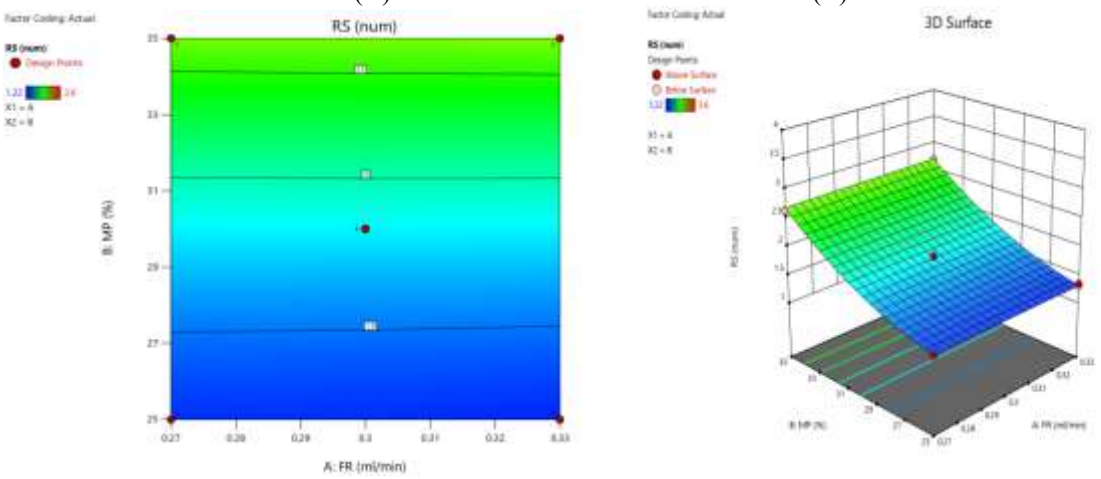
A quadratic equation depicting the relationship between the independent variables and the resolution factor (Y1) follows.

Y1 (Resolution Factor) = 1.80 – 030013X1 + 0.6834 X2 + 0.0113X1X2 – 0.0025X12 +0.2061X22

One can employ the equation derived from encrypted variables to forecast the behavior of each element in particular conditions. The states of the factors are represented as 1 for high and -1 for low by default. The encrypted equation, by the comparison of its components, elucidates the relative significance of the factors. The findings of the analysis of variance (ANOVA) are displayed in Table 4. An F-value of 1278.73 indicates that the model is statistically significant. A random occurrence of an F-value of this size is less likely than 0.01%. When the p-value falls below 0.0500, we say that

the model terms are significant. Figure 2B shows a three-dimensional response surface chart, while Figure 2A shows a two-dimensional plot, both of which show how the independent variables have an effect.

(A) (B)



'Fig. 2 The two-dimensional contour plots (A) and the three-dimensional response surface plots associated (B) showing the effect of independent variables on the resolution factor (Y1)'

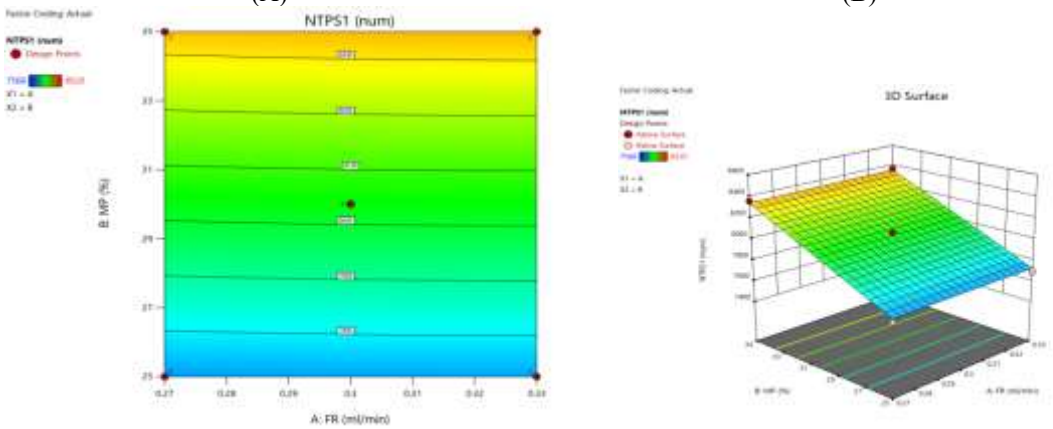
5.3.2 Impact of explanatory factors on Vildagliptin (Y2) theoretical plate number:

Various independent parameters impact the theoretical plate of VGT (Y2), as shown by the quadratic equation below.

Y2 theoretical Plates of VGT = 8036.88 +4.72X1 +313.03X2 +0.5000X1X2 -2.38X12 -1.32X22

The equation utilizing encrypted factors can be employed to forecast the reactions of each ingredient in particular settings. The states of the factors are represented as 1 for high and -1 for low by default. The encrypted equation makes it easy to compare and contrast the component parts and determine their relative importance. If you look in Table 5, you can see the outcomes of the ANOVA. Statistical proof is provided by the model's F-value of 283.30. A random occurrence of an F-value of this size is less likely than 0.01%. When the p-value falls below 0.0500, we say that the model terms are significant. Part of the model that is absolutely necessary is B. For values less than 0.1000, the model terms do not have any meaningful impact. One can navigate the design domain by employing this paradigm. Figure 3A and B show, in two-dimensional and three-dimensional response surface plots, respectively, how the independent factors affected the theoretical VGT plates.

(A) (B)



'Fig. 3 The 2D contour plots (A) and the associated 3D response surface plots (B) showing the effect of independent variables on the on the tailing factor of Vildagliptin (Y2)'

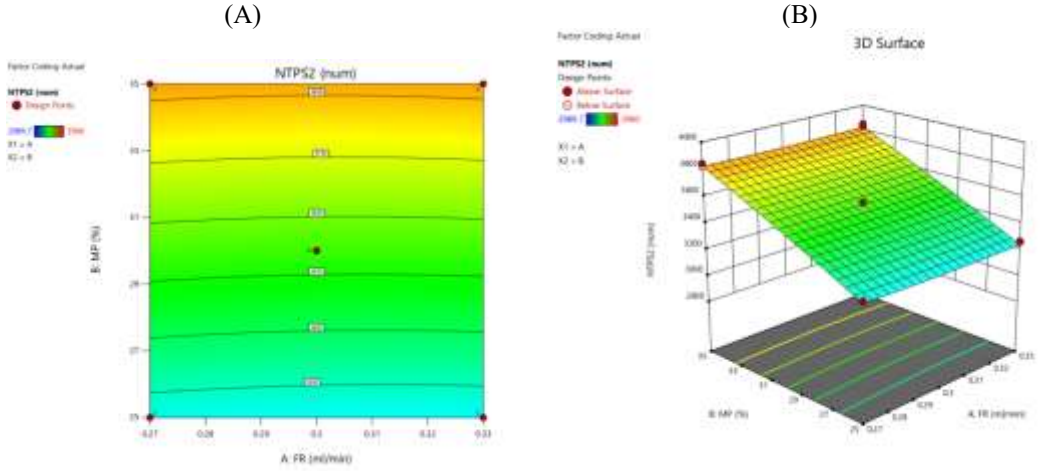
5.3.3 Effects of explanatory factors on the theoretical plate count of pioglitazone (Y3):

The theoretical PGT (Y3) plate can be described by this quadratic equation, which details the possible impacts of independent factors.

Y3 theoretical Plates of PGT = 3541.83 - 3.94X1 +289.27X2 + 3.12X12 -12.97X22

The equation utilizing encrypted factors can be employed to forecast the reactions of each ingredient in particular settings. The states of the factors are represented as 1 for high and -1 for low due to neglect. Results from the analysis of variance (ANOVA) pertaining to friction are displayed in Table 6. A high Model F-value (555.45) suggests a robust model. A chance occurrence of less than 0.01% is required to produce an F-value of this magnitude. When the p-value falls below 0.0500, we say that the model terms are significant. Here, B and $B^2$are the main terms in the model. By using the encrypted equation, it is easier to compare the components and determine their relative importance. The influence of independent parameters on the theoretical PGT plates is shown in Figure 4A, a two-dimensional plot, and Figure 4B, a three-dimensional response surface plot.

(A) (B)



'Fig. 4 The two-dimensional contour plots (A) and the associated three-dimensional response surface plots (B) showing the effect of independent variables on the theoretical plates of pioglitazone (Y3)'

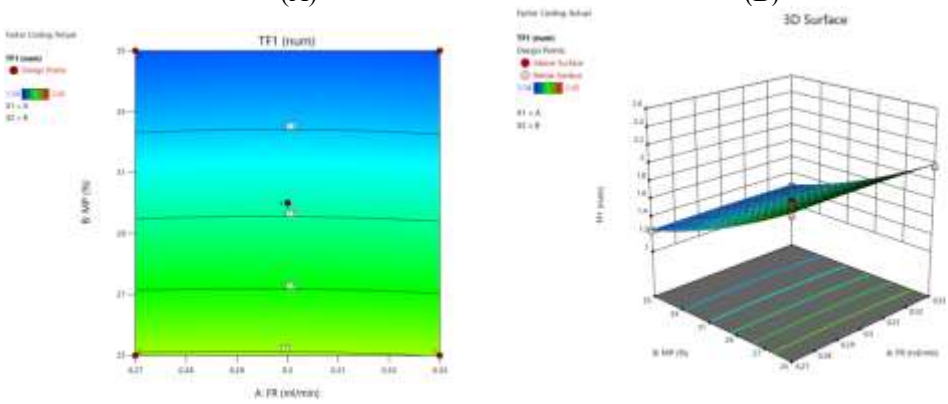
5.3.4 Vildagliptin (Y4) tailing factor as affected by independent variables:

After that, the connection between the independent variables and the VGT tailing factor is described by a quadratic equation.

Y4 Tailing factor of VGT = 1.57 - 0.0029X1 - 0.3766X2 + 0.0025X1X2 – 0.0095X12 + 0.0701X22

One can employ the equation derived from encrypted variables to forecast the reaction of each ingredient under particular conditions. The states of the factors are represented as 1 for high and -1 for low by omission. Analysed in Table 7 are the outcomes of the ANOVA. An F-value of 521.87 indicates statistical significance for the model. A random occurrence of an F-value of this size is less likely than 0.01%. When the p-value falls below 0.0500, we say that the model terms are significant. Here, B and B²are the key terms in the model. By comparing its parts, the encrypted equation reveals which aspects are more important. Figures 5A and 5B illustrate the influence of several parameters on the trailing factor of VGT, presenting analogous 2D plots and distinct 3D response surfaces.

(A) (B)



'Fig. 5 The associated 2D contour plots (A) and The 3D response surface plots (B) showing the effect of independent variables on the on the tailing factor of Vildagliptin (Y4)'

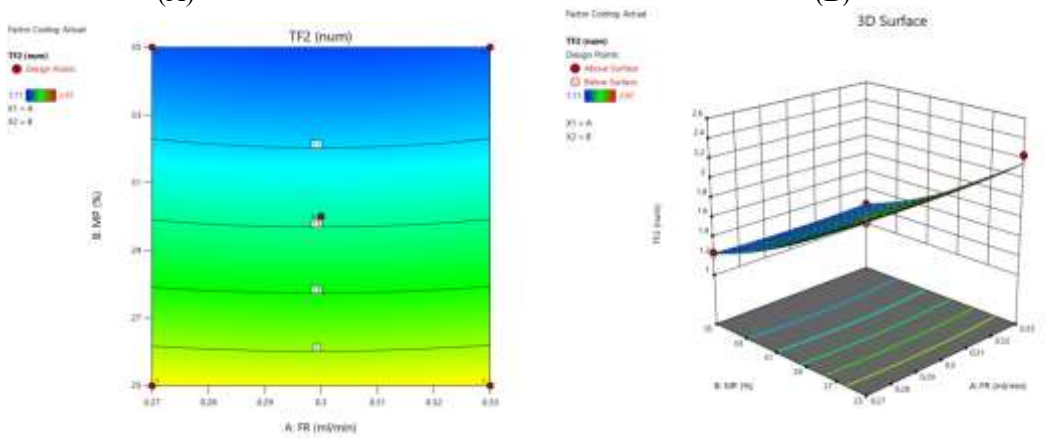
5.3.5Tailing factor of Pioglitazone (Y5) affected by independent variables:

For each given set of independent variables, the PGT theoretical plate can be described by the quadratic equation that follows.

Y5 Tailing factor of PGT = 1.57 + 0.0007X1 – 0.4611X2 – 0.0012X1X2 + 0.0205X12 + 0.0983X22

The equation derived from encrypted factors can be employed to forecast the reactions of each element in particular settings. The states of the factors are represented as 1 for high and -1 for low by omission. The outcomes of the ANOVA may be seen in Table 8. A large model is indicated by the Model F-value of 139.96. There is less than a 0.01% chance that an F-value of this size could have happened by chance alone. If the p-value is less than 0.0500, then the model terms are considered significant. The crucial parameters of the model here are B and B². If the values are less than 0.1000, the model terms are considered negligible. On the other hand, if your model contains a lot of irrelevant terms (aside from those related to size), model reduction could improve it. The encrypted equation makes it easy to compare and contrast the components and determine their relative importance. The influence of several variables on the PGT theoretical plates is shown in Figures 6A and B, which comprise both 2D figures 10 and 3D response surface plots.

(A) (B)



'Fig. 6 The 2D contour plots (A) and the associated 3D response surface plots (B) showing the effect of independent variables on the on the tailing factor of Pioglitazone (Y5)'

'Table 3: Fitting to different polynomial replies for answers Y1 through Y5 using regression analysis'

'model	SD	R2	Adjusted R2	Predicted R2	PRESS	Remarks
Y1						
Linear	0.1942	0.9087	0.8979	0.8501	1.05	
2FI	0.2000	0.9088	0.8917	0.8482	1.07	
Quadratic	0.0331	0.9978	0.9970	0.9922	0.0547	Suggested
Cubic	0.0267	0.9988	0.9981	0.9765	0.1651	
Y2						
Linear	28.01	0.9901	0.9890	0.9847	20689.49	Suggested
2FI	28.86	0.9901	0.9883	0.9825	23609.52	
Quadratic	30.74	0.9902	0.9867	0.9647	47459.96	
Cubic	19.08	0.9968	0.9949	0.9708	39485.37	
Y3						
Linear	23.50	0.9919	0.9909	0.9864	15634.33	
2FI	24.12	0.9919	0.9904	0.9854	16808.68	
Quadratic	20.32	0.9950	0.9932	0.9834	19186.20	Suggested
Cubic	20.66	0.9956	0.9930	0.8930	1.23E+05	
Y4						
Linear	0.0711	0.9575	0.9525	0.9268	0.1480	
2FI	0.0733	0.9575	0.9496	0.9265	0.1487	
Quadratic	0.0278	0.9947	0.9928	0.9799	0.0406	Suggested
Cubic	0.0276	0.9955	0.9929	0.8628	0.2775	
Y5						
Linear	0.1094	0.9345	0.9268	0.9027	0.3025	
2FI	0.1128	0.9345	0.9223	0.8899	0.3419	
Quadratic	0.0660	0.9804	0.9734	0.9189	0.2520	Suggested
Cubic	0.0498	0.9904	0.9848	0.6746	1.01'	

Y1: Resolution Y2: Theoretical plates of VGT, Y3: Theoretical plates of PGT, Y4: Tailing factor of VGT, Y5: Tailing factor of PGT

'Table 4: Analysis of Variance (ANOVA) test results and adequate precision for varies replies'

	'Y1		Y2		Y3		Y4		Y5	
	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value
Model	1278.73	<0.0001	283.30	<0.0001	555.45	<0.0001	521.87	<0.0001	139.96	<0.0001
X1	0.0199	0.8897	0.3219	0.5795	0.5146	0.4850	0.1519	0.7026	0.0017	0.9679
X2	5822.33	<0.0001	1416.08	<0.0001	2767.98	<0.0001	2511.72	<0.0001	667.05	<0.0001

X1X2	0.9243	0.3527	0.0021	0.9640	0.1892	0.6702	0.0648	0.8027	0.0029	0.9580
X12	0.0827	0.7779	0.0873	0.7720	1.94	0.1857	1.70	0.2133	1.40	0.2563
X22	564.42	<0.0001	0.0268	0.8722	5.93	0.0289	92.64	<0.0001	32.28	<0.0001
Adequate precision	126.7987		62.5331		87.4275		83.2822		42.9185’	

X1 and X2 coded levels of independent variables; X1X2 are interaction terms; X12 and X22 quadratic terms

5.3.6 Choosing the most suitable chromatographic parameters:

We identified the optimal chromatographic settings through the numerical optimization method integrated into the Design Expert® tool. To isolate PGT and VGT, a mobile phase was formulated using 30.2 volumes of acetonitrile and 69.8 volumes of a solution containing 7.8 g of dibasic sodium phosphate in water. The supplementary chromatographic parameters, in accordance with the program's directives, included a pH of 4.4 adjusted using monobasic sodium phosphate, 0.3 mL/min of inflow, 30 °C for the column, and 212 nm for UV detection. The UPLC system proved to be suitable for achieving outstanding chromatographic results after 50 µg/mL of VGT and 15 µg/mL of PGT were added. For each possible answer, Table 5 shows the expected and observed values as well as the residual percentage. The chromatographic parameters needed to separate PGT and VGT were determined using a QbD design, which produced residual values ranging from 0.320 to 2.53. To ensure the proposed approach worked, we fine-tuned the chromatographic parameters. With a minimal residual error rate of 11, the results show that the underlying composite design was robust.

‘Table 5: Optical chromatographic conditions yielded the following expected and observed values of responses’

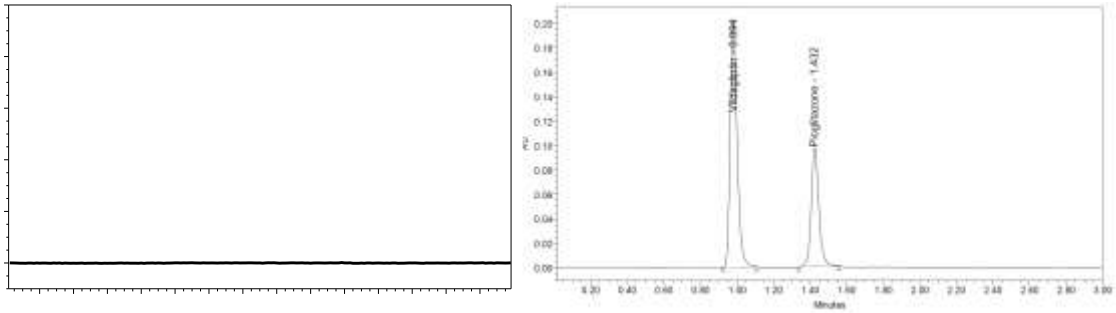
‘Response	Predicted Value	Observed value	Residual values (%)
Retention time of VGT (min)	0.9806	0.9943	0.0001
Retention time of PGT (min)	0.9945	0.9984	0.0003
Resolution factor	0.6341	0.8444	0.0139
Theoretical plates of PGT	0.9863	0.9963	1.302
Tailing factor PGT	0.9203	0.9772	0.0001’

5.3.7 Validation of the Method

Table 6 presents a detailed enumeration of the attributes that signify a system's appropriateness, along with the corresponding acceptance criteria. The theoretical plate count, resolution factor, tailing factor, and relative standard deviation (RSD) percentage are examined in relation to the reference values. The theoretical plate counts for VGT are 3052 ± 45, but for PGT, they are 8349 ± 15. Moreover, the retention durations and peak regions of both pharmaceuticals are below 1. PGT possesses a trailing value of 1.19, whereas VGT exhibits a trailing factor of 1.24. The absolute RSD is awarded two points, whilst the resolution factor is granted an additional two points. The results are illustrated in the image. Figure 8 illustrates the linearly optimized chromatograms for PGT and VGT. In contrast to VGT, while PGT's concentration ranges from 3.75-22.5 µg/mL, while the former has a range of 12.5-75 µg/mL. A regression coefficient (r^2) of 0.9998 was obtained from the analysis of the effects of PGT on the variables x and y. With a VGT regression coefficient (r^2) of 0.9994, the results for LOQ and LOD were different between PGT and VGT, according to the regression equation $y = 7362.2X + 2108.2$. The levels of PGT and VGT were found to be 0.68 µg/mL and 0.44 µg/mL, respectively, whereas the levels of VGT were found to be 1.32 µg/mL. Table 7 shows that both PGT and VGT are very accurate both within and across days, with a relative standard deviation of no more than 2. Table 8 shows that there was a significant difference between the recovery rates of VGT (100.98%) and PGT (99.09%). Figure 10 shows how accurate the method is. Conventional methods were able to efficiently separate PGT and VGT from the expression excipients because, unlike the blank and placebo samples, they did not co-elute throughout the retention period. The robustness data results are displayed in Table 9. Relative standard deviations for the peak area and retention duration variations were less than 2. The proposition plates, the trailing factor, and the resolution factor—all indicators of system efficacy—remained unchanged. The commercially available formulation consisted of a specified ratio of 100.39 mg PGT to 100.14 mg VGT, as determined in the drug assay. An examination of the peak areas before and after 24 hours indicates that PGT and VGT maintain consistent results.

‘Table 6: System suitability test (n=6)’

‘Parameter	VGT	PGT	Acceptance Criteria
Retention time	0.994 ± 0.005	1.432 ± 0.005	–
Peak area	368455	102668	< 1 for n ≥ 5
RSD % of peak area	1.0	1.1	–
Theoretical plates (N)	8349 ± 24	3052 ± 65	< 1 for n ≥ 5
Tailing factor (T)	1.24 ± 0.02	1.19 ± 0.005	> 2000
Resolution (Rs)	0	2.8	≤ 2.0’



'Fig. 7 Chromatograms of placebo (A) and standard vildagliptin and pioglitazone (B) showing the specificity of the established UPLC method'

'Table 7 linearity curve of vildagliptin and pioglitazone'

'S.no	Conc (ug)	vildagliptin Peak area	Conc(ug)	pioglitazone Peak area
01	0	0	0	0
02	12.5	90334	3.75	26916
03	25	184582	7.5	53806
04	37.5	284469	11.25	80071
05	50	373741	15	105468
06	62.5	461697	18.75	134086'

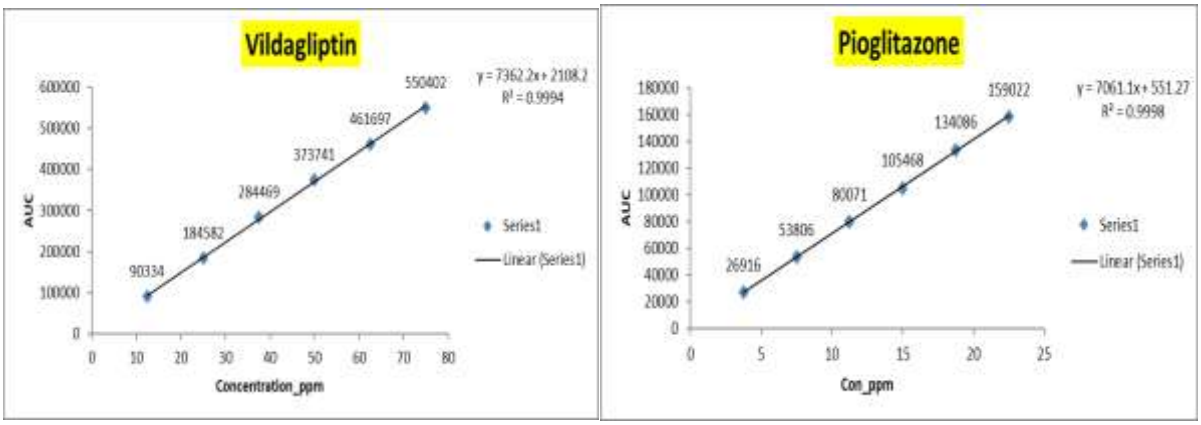


Fig 8 Calibration curve of vildagliptin and PGT

'Table 8 Recovery studies of vildagliptin and pioglitazone'

'Compound	Contents (ug)	Quantity added (ug)	Recovered amount (ug)	Recovery (%)	%RSD
vildagliptin	50	25	24.9	99.7	
50%	50	25	25.1	100.3	0.65
	50	25	24.7	99.0	
100%	50	50	50.5	100.9	
	50	50	50.5	101.0	0.18
	50	50	50.3	100.6	
150%	50	75	75.3	100.4	
	50	75	75.7	100.9	0.26
	50	75	75.4	100.6	
PGT	15	7.5	7.54	100.59	
50%	15	7.5	7.51	100.10	0.66
	15	7.5	7.45	99.28	
100%	15	15	14.91	99.40	
	15	15	15.07	100.45	0.60
	15	15	15.07	100.44	
150%	15	22.5	22.52	100.07	
	15	22.5	22.52	100.09	0.19'
	15	22.5	22.44	99.75	

'Table 9 Intraday and interday precision of vildagliptin and pioglitazone'

'S.no	VGT		PGT	
	Intra-day Precision	Inter-day Precision	Intra-day Precision	Inter-day

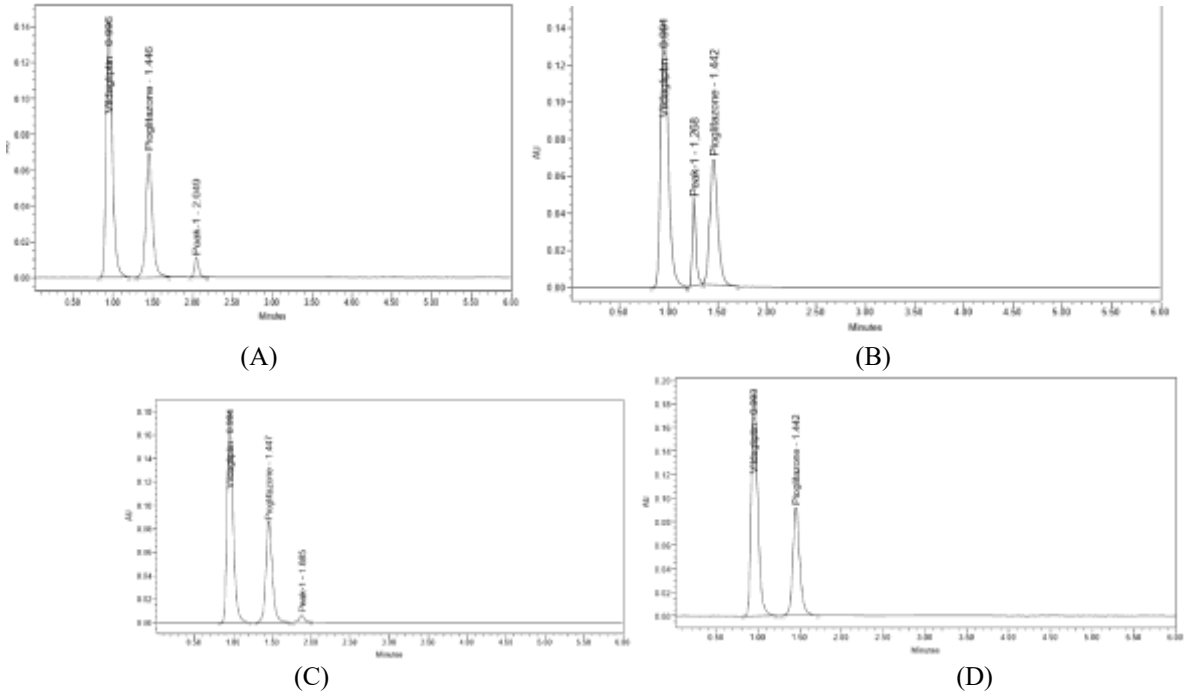
				Precision
01	368589	369474	104368	107755
02	363858	369848	107657	105386
03	364057	364385	104737	105437
04	364947	359848	106363	105437
05	364954	360485	104737	103736
06	362748	364957	106548	105474
Mean	364526	364833	105735	105538
Std.dev	1299.5	4258.2	1312.3	1281.4
% RSD	0.4	1.2	1.2	1.2’

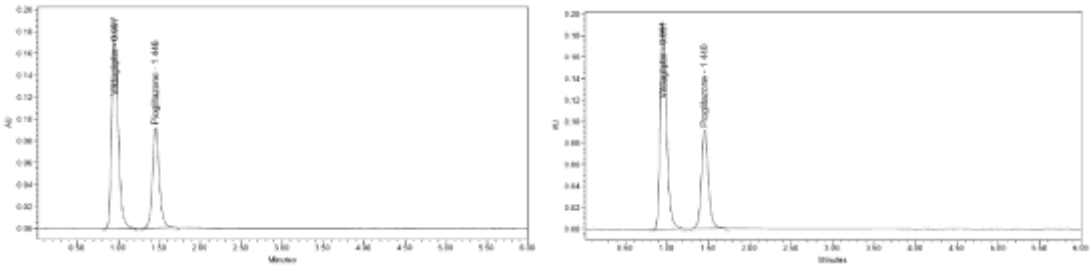
5.3.8 Investigations on forced deterioration [36-44]

The results of the forced declination trials conducted on PGT and VGT, respectively, under varying stress circumstances are shown in Figure 9 and Table 10, respectively. Both PGT and VGT maintained a fall rate below 2% across all test conditions, which ranged from acidic to alkaline, hot to neutral. VGT declined by 3.92 percent, whereas PGT exhibited a singular peak at 1.885 minutes during oxidative degradation. The degradation rate for PGT was 2.55% following photolytic deterioration, whereas VGT exhibited a peak degradation time of 0.994 minutes; the degradation rates for the two materials were 1.52 and 1.447 minutes, respectively. No variation was observed in the degradation peaks.

‘Table 10 Stability studies of vildagliptin and pioglitazone’

‘Stress condition	**Treatment**	**% Degradation**	
		VGT	PGT
Acid hydrolysis	2N HCl, 60 °C for 30 min	4.47	4.16
Alkaline hydrolysis	2N NaOH, 60 °C for 30 min	6.91	6.44
Oxidative degradation	3% H2O2 60 °C for 30 min	3.92	3.69
Thermal degradation	105 °C for 6 h	0.90	1.61
Photolytic degradation	UV light of 1.2 million lux hours for 7 days	1.52	2.55
Neutral hydrolysis	Water at 60 °C for 6 h	1.52	0.91’





(E) (F)

'Fig. 9 Vildagliptin and pioglitazone degradation profiles under various stress conditions are displayed in chromatograms as acid (A), alkali (B), oxidative (C), dry heat (D), neutral (E), and photolytic degradation (F)'

6 DISCUSSIONS: SUBJECT UNDER CONSIDERATION

In this study, a new RP-UPLC method was developed for the evaluation of concentrations of AQbD-based PGT and VGT. The chromatographic parameters of the proposed method were fine-tuned using the Design-expert® program (Stat-Ease Inc., USA) in a three-part central composite design (CCD) that progressed through five stages. Utilising polynomial equations, 3D response graphs, and 2D figure plots, the effects of temperature, input rate, and methanol concentration on retention duration, resolution factor, number of theoretical plates, and trailing factor were investigated. To determine if the model and its parameters were statistically significant, we ran an analysis of variance. The next step was to find the right chromatographic parameters by using a numerical optimisation method. The most important factors affecting the retention periods of PGT and VGT, respectively, were found to be temperature and flow rate, according to the polynomial equations and three-dimensional response plot. More important than temperature or flow rate was the acetonitrile concentration in the mobile phase for determining the resolution factor. The total theoretical plates for both medications were found to be most affected by the flow rate. After accounting for the tailing effect, the results showed that flow rate affected VGT more than acetonitrile % affected PGT. With little residual error and strong correlation between predicted and actual findings, the QbD model successfully identified the suitable chromatographic parameters for VGT and PGT estimations.

Analytical techniques must be validated to guarantee their robustness and appropriateness for the intended application. The RP-UPLC method developed conforms to the standards established by ICH Q2 R1. Prior to initiating the study, we conducted a system suitability test to ensure the selected chromatographic system would function correctly. It is essential to do this test during the development of a process. The chromatographic system was deemed adequate as all measurements were within the permissible ranges. Theoretical plates, trailing factors, resolution factors, probability, standard deviation compared to other parameters, peak area, retention time, etc, were included in the analysis. Correlation coefficient (r2) values exceeding 0.9999 signify a robust association between the two medications and a linear relationship within the proposed concentration range. The proposed approach demonstrated sufficient sensitivity to identify the prescription dosage forms of PGT and VGT, exhibiting reduced limits of detection (LODs) and limits of quantitation (LOQs). "Analytical precision" denotes the extent to which two sets of results concur under similar experimental conditions. The three distinct levels of intraday and interday precision, each exhibiting percentage RSD values under 2%, demonstrated the superiority of the proposed strategy. Assessing the degree to which the experimental outcome aligns with the true value is a vital component of recovery research. The experimental and actual results for VGT (from 99.65% to 100.71%) and PGT (from 100.98% to 99.08%) were notably consistent. When an analytical process can identify the target analyte in commercially available formulations without interference from pollutants, degradation agents, or excipients, we say that the procedure is specific. One way that VGT and PGT stand out from other pharmaceuticals is that their retention times are not altered by co-eluting interference peaks, according to the accepted method for evaluating these medications from pharmaceutical dose forms. We contend that a method is resilient if it can uphold consistency despite significant variations in chromatographic parameters. This feature determines the method's reliability in real-world conditions. The approach's robustness was demonstrated by low % RSD results, and system suitability parameters were not considerably altered by the intended modifications to testing conditions. The current RP-UPLC technique enables the quantification of PGT and VGT concentrations in commercial formulations. The assay findings, as shown on the label, may range from 100.14% to 100.39%. The stability research indicated that the VGT and PGT solutions exhibited no alterations after one day of storage at ambient temperature.

The efficacy of pharmacological and other therapeutic products can only be determined through stability testing. The method demonstrates its capacity to evaluate VGT and PGT stability by examining their degradation under various stress conditions. In photolytic or oxidative conditions, both drugs had remarkable efficacy; however, they proved useless in neutral, acidic, or alkaline settings. The results demonstrate that the current methodology is stable.

7 CONCLUSIONS

A RP-UPLC system was optimized to quantify PGT and VGT through a systematic approach focused on quality by design, particularly utilizing central compound design. The optimal chromatographic settings for both VGT and PGT are outlined below. These are some of the measured values: 0.3 mL/min flow rate, 30 °C column temperature, 212 nm UV discovery wavelength, 30.2 litres acetonitrile, and 69.8 volumes of 0.1 OPA. The system reported 1.432 twinkling peaks for PGT and 0.994 for VGT, respectively. The system's strength, precision, sensitivity, and awareness were confirmed through verification in accordance with ICH standards. During the forced degradation testing, both VGT and PGT exhibited

degradation peaks along with distinctly defined peaks. The findings of this study indicate that AQbD may serve as an effective instrument for enhancing existing strategies and demonstrate its capability in accurately identifying pharmaceutical dosage forms of gliclazide and sitagliptin.

Abbreviations
'Analytical Quality by Design (AQbD)
Quality by Design (QbD)
Programmed Death Receptor-1(PD-1)
Reverse Phase Ultra Performance Liquid Chromatography (RP-UPLC)
Central Composite Design (CCD)
Liquid Chromatography-Mass Spectrometry (LC-MS/MS)
Liquid Chromatography-Mass Spectrometry/High Resolution Mass Spectrometry (LC-MS/HRMS)
Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)
Ultra High-Performance Liquid Chromatography/Ultra Violet-Heated Electro Spray Ionization (UHPLC/UV-HESI)
Ultra Violet (UV)
Tunable Ultra-Violet (TUV)
Limit Of Detection (LOD)
Limit of quantification (LOQ)
Food and Drug Administration (FDA)
Bridged Ethylene Hybrid (BEH)
Hydro Chloric Acid (HCl)
Sodium Hydroxide (NaOH)
Hydrogen Peroxide (H2O2)
Standard Deviation (SD)
Relative Standard Deviation (RSD)
Analysis of Variance (ANOVA)
Three Dimensional (3D)
Coefficient of Variance (CV)
Predicted Residual Error Sum of Squares (PRESS)'

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Human and animal rights
Research involving plants should include a declaration that specifies the relevant laws and regulations at the state, federal, or international level, along with any required authorisations or licences: Not applicable.

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