Development and Validation of a Stability Indicating Analytical Method for the Estimation of Gliclazide and Sitagliptin in Bulk Drugs and Tablet Dosage Forms by RP-UPLC: An Application of the Quality-by-Design Approach

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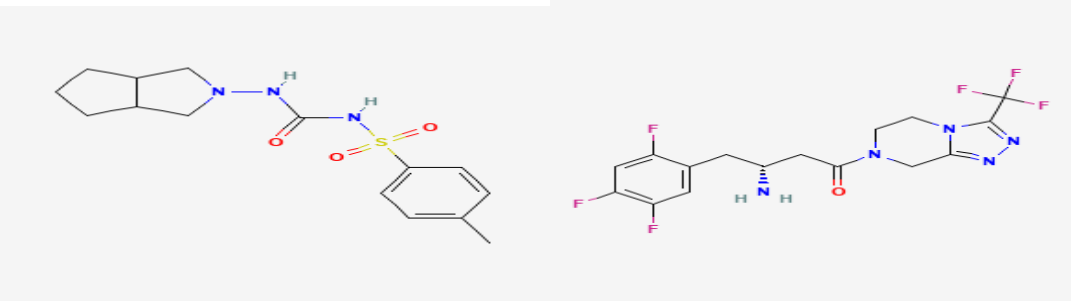
Analytical Quality by Design, Sitagliptin and Gliclazide, Central Composite Design, Reversed-Phase Ultra Performance Liquid Chromatography.

ABSTRACT
Sitagliptin and gliclazide are the active ingredients in OpdualagTM. A highly selective DPP-4 inhibitor, sitagliptin is thought to work in type 2 diabetics by delaying the inactivation of incretin hormones, which increases their concentration and lengthens their duration of effect. Sulfonyl urea mostly enhances insulin secretion from pancreatic β-cells by inhibiting ATP-sensitive K+ channels, leading to depolarisation and Ca2+ influx. Reduced serum glucagon levels and enhanced insulin binding to receptors and target tissues are the mechanisms by which it exerts its effects. Objective: This study aims to create and evaluate an RP-UPLC technique for the detection of sitagliptin and gliclazide in pharmaceutical products by applying AQbD principles. Method: Findings By utilising the Design-expert® programme, we were able to execute a central composite design (CCD) consisting of three components arranged in five distinct layers, which greatly improved the chromatographic conditions. One method that oversees the whole analytical procedure life cycle—from method design and optimisation to validation and continual improvement—is Analytical Quality by Design (AQbD). Results: The retention times for sitagliptin were 1.269 minutes and for gliclazide they were 1.572 minutes. The limits of detection (LOD) and quantitation (LOQ) for Sitagliptin and Gliclazide were 0.19 µg/mL, 0.56 µg/mL, 0.28 µg/mL, and 0.86 µg/mL respectively. The recovery percentages for sitagliptin and gliclazide were found to be 99.15% to 100.54% and 99.93% to 100.58%, respectively. Both drugs were found to be susceptible to oxidative and photolytic breakdown in the forced degradation studies. Conclusions A novel RP-UPLC method has been developed for the quantitative detection of sitagliptin and gliclazide in pharmaceutical formulations, utilising the AQbD-based methodology. This approach is straightforward, rapid, precise, particular, and stable-indicating.

1. Introduction

The compound sitagliptin is a 1:1 mixture of pyrazine phosphate and 7-[(3R)3-amino] butyl of 1-oxo-4-(2,4,5-trifluorophenyl)5, 6, 7, and 8 tetrahydro Trifluoromethyl-3-1,2,4-triazolo[4,3-a] monohydrate. In type 2 diabetics, the highly specific DPP-4 inhibitor sitagliptin increases the concentration and duration of action of incretin hormones by postponing their inactivation. It dissolves in water and N, N-diethyl formamide but has poor solubility in methanol, ethanol, acetone, and acetonitrile; it is insoluble in isopropanol and isopropyl acetate. This paper details an RP-UPLC method that is simple, sensitive, and accurate for estimating the two drugs in their combination tablet dose form at the same time. Pictured in Figure 1(A) is the structure of sitagliptin [6,11].

One of the classes of drugs known as sulfonyl ureas is gliclazide. Its chemical name is 1-(hexa hydroxy clopenta,pyrrol-2(1H-yl))-3-[(4-methyl phenyl) sulfonyl urea. By inhibiting ATP-sensitive K+ channels, sulfonyl urea mainly induces depolarisation and Ca2+ influx, which in turn promotes the insulin release from the pancreatic β-cells. Additionally, it enhances insulin binding to receptors and target tissues while decreasing serum glucagon levels [12]. The structure of Gliclazide is shown in Figure 1(B).



The structures of sitagliptin and gliclazide are shown in Figure 1 (A) and Figure 1 (B), respectively.

An extension of the Quality by Design (QbD) concept [1,4], Analytical Quality by Design (AQbD) ensures the precision of analytical operations in the pharmaceutical sector. This method allows for a more thorough comprehension of the method's primary parameters and how they impact analytical results, leading to more efficient and effective analytical development and validation procedures. Not only can AQbD help find the right chromatographic conditions to measure analytes, but it also helps optimise the most important components and the effects of their interactions. The most common experimental layout for response surface methods is a central composite design, which maximises chromatographic parameters. Using a small number of experimental trials, these strategies produce a large amount of data at a low cost.

The literature analysis found that there are few analytical methods available for the detection of sitagliptin in plasma and pharmaceutical dose forms, both when used alone and in combination with other medicines. Some examples of these methods are RP-UPLC, LC-MS/MS, ELISA, LC-MS/HRMS, and UPLC-MS/MS, as well as the UHPLC/UV-(HESI/OrbitrapTM) MS approach [7]. The simultaneous evaluation of sitagliptin and gliclazide in OpdualagTM formulations is possible using an RP-UPLC method. The AQbD principles can be used to estimate gliclazide and sitagliptin, although no such established analytical approach exists at this time [20].

An RP-UPLC method for the estimation of sitagliptin and gliclazide in pharmaceutical products based on AQbD principles (central composite design) was developed and optimised in the present study [12,13].

2. Methods and Equipment's

2.1. Chemicals

As reference standards, we acquired Sitagliptin (99.9% w/w) and Gliclazide (99.9% w/w) API samples from Zydus Cadila Health Care Ltd. in Secunderabad. The research used the SR formulation of Cyblex S 30XR Tablets.

2.2. Chemicals and reagents

Potassium dehydrogenate ortho phosphate buffer, methanol, acetonitrile, distilled water, triethyl ethylamine, and ortho-phosphoric acid. Rankem Chemicals of Haryana, India, is the supplier of the aforementioned chemicals and solvents. An ultrapure supply of water was purchased by Merck Millipore of India from the Millipore Direct-Q®3 ultraviolet water purification system. Analytical grade was met by all of the other compounds and reagents.

2.3. Equipment

This study made use of the Waters Acquity UPLC System, which features an auto sampler connected with Empower 3, a TUV detector, and binary pumps. An ultraviolet-visible spectrophotometer (PG Instruments T60) with 2 mm and 10 mm of specific bandwidth, matching quartz cells, and UV Win 6 software were utilised in the experiment. A pH metre, an ultra sonicator, a Millipore BM2EA9672R, an LG refrigerator, and BVK Enterprises, India, were among the other instruments used.

2.4. Composition of solutions

In a 0.1% OPA buffer solution:

1 millilitre of orthophosphoric acid in 900 millilitres of water. Once you've adjusted the pH to 4.6 ± 0.5 with 0.01 N potassium hydroxide, dilute it with 1000 mL of water.

2.5. During the mobile phase

The mobile phase consists of 30.6 volumes of acetonitrile and 69.4 litres of buffer that has been filtered properly.

2.5.1. To dilute

The solubility analysis led to the selection of a 60:40 mixture of acetonitrile and water as the diluent.

2.6. Preparation of standard stock solutions

Mix 10 milligrams of sitagliptin and 3 milligrams of gliclazide in a 25-millilitre clean and dry volumetric flask after they were weighe2.7d precisely. Before sonicating for 10 minutes, add 10 mL of diluent to ensure complete dissolution. In order to achieve sitagliptin and gliclazide concentrations of 400 µg/mL and 120 µg/mL, respectively, the volume was raised to 25 mL using diluent. Various quantities of Gliclazide (3, 6, 9, 12, 15, and 18 µg/mL) and Sitagliptin (10, 20, 30, 40, 50, and 60 µg/mL) were achieved by diluting portions of each stock solution with the diluent to create working standard solutions.

2.7. Preparation of stock solution:

The weight of one tablet (containing Gliclazide (30 mg) and Sitagliptin (100 mg)) was ascertained by weighing ten pills and determining their average weight. The average weight was 248. After adding 24 mg to a 50 ml volumetric flask, 25 ml of diluents, and 25 minutes of sonication, the mixture was diluted with diluent and filtered using HPLC filters with concentrations of 600µg/ml of Gliclazide and 2000µg/ml of Sitagliptin. The final solution was then used in its commercial formulation to undertake sitagliptin and gliclazide assays.

3. Optimisation of methods through experimental design

The chromatographic conditions of the methodology that was established were optimised using Design-expert® software (Version 11.1.0.1, Stat-Ease Inc., USA). This software utilised a central composite design (CCD) with three elements at five levels (− α, − 1, 0, + 1, and + α). The mobile phase was chosen based on preliminary studies using acetonitrile and 0.1N OPA buffer. Temperature (X3), flow rate (X1), and percentage mobile phase (X2) were chosen as the independent variables, while resolution factor (Y3), number of theoretical plates of Gliclazide (Y5), number of theoretical plates of Sitagliptin (Y4), tailing factor of Gliclazide (Y6), and tailing factor of Sitagliptin (Y7) were chosen as the dependent variables (Table 1). The Design Expert® software recommended twenty iterations based on the primary composite design. All trials utilised a standard concentration of 60 µg/mL of sitagliptin and 18 µg/mL of gliclazide [16,17].

Here are the key factors in the composite design and their respective levels:

A set of independent factors

Name	-α	-1	0	+1	+α
X_1 Flow rate (ml/min)	0.20	0.2095	0.3	0.35	0.3505
X_2 %Mobile phase	20.00	20.59	30.00	40.00	40.41
X_3 Temperature 0 ^{C}	20.00	20.59	30.00	35.00	35.00

Factors that are dependent

Y1: Sitagliptin retention time; Y2: Gliclazide retention time; Y3: Resolution factor; Y4: Gliclazide number of theoretical plates; Y5: Sitagliptin number of theoretical plates; Y6: Tailing factor of sitagliptin; Y7: Tailing factor of Gliclazide

4. Validation of the Method

The new RP-UPLC method was validated for precision, accuracy, linearity, LOD, LOQ, system adaptability, and robustness [2], in compliance with ICH Q2 (R1) standards [2]. A total of six replicates of gliclazide (18 µg/mL) and sitagliptin (60 µg/mL) standard solutions are injected during the system suitability test. They check their theoretical plates, peak area, resolution, tailing factor, and percent RSD of retention time. The linearity of the technique could be confirmed by plotting the calibration curve of peak area against concentration at six concentrations of Sitagliptin (10-60 µg/mL) and Gliclazide (3-18 µg/mL) using working standard solutions. LOD was calculated as 3.3 × σ/S and LOQ as 10 × σ/S, using the slope (S) and standard deviation (ғ) of the calibration curve, respectively. The method's correctness was confirmed by variance studies that were performed

both within and between days. Gliclazide and Sitagliptin were evaluated three times daily for repeatability at three distinct concentrations (3, 9, and 18 µg/mL) and for inter-day accuracy at three different concentrations on three separate days. For the purpose of evaluating accuracy, standard solutions containing 50, 100, and 150 percent Sitagliptin and Gliclazide were added to previously examined samples of these drugs [14,15,18].

One way to ensure that the technique was particular was to compare the representative chromatograms of the Sitagliptin/Gliclazide standard solution, blank, and placebo. The ideal chromatographic parameters, such as flow rate (0.1 and 0.3 mL), methanol percentage (± 5% in mobile phase), and column temperature (± 3 °C), were intentionally adjusted in order to assess robustness.

4.1. Consistency of the sample solution

The solution containing sitagliptin and gliclazide was tested for stability by storing samples in a volumetric flask under standard ambient laboratory conditions for 24 hours. The original data was compared to the calculated peak areas and retention times of sitagliptin and gliclazide after 24 hours [12].

4.2. Investigations on forced deterioration

Gliclazide and sitagliptin were subjected to studies that examined their forced degradation under different stress situations [3,5]. To expose the sitagliptin and gliclazide solutions to acid, several stress conditions were employed, such as 2N HCl at 60 °C for 30 minutes. The Sitagliptin and Gliclazide solutions were subjected to a variety of degradation processes, including acid (60% H2O2 at 60 °C for 30 min), alkaline (2N NaOH at 60 °C for 30 min), dry heat (105 °C for 6 h), neutral (water at 60 °C for 6 h), and photolytic (7 days of UV radiation). Gliclazide was diluted to 18 µg/mL and Sitagliptin to 60 µg/mL after exposure. Chromatograms were taken to evaluate the sample's stability after injecting five microliters of each solution into the apparatus [8].

4.3. Evaluation using statistical methods

We reported the data using the mean plus or minus the standard deviation. The regression coefficient, mean, standard deviation, and % RSD were calculated using Excel. The model and its terms were determined to be statistically significant using analysis of variance (ANOVA). Each term and model were deemed significant if the p-value was 0.05 or lower [10].

5. Results and Discussion

Studies on the development of methods

In order to create the LC method for compound separation, a variety of solvents (e.g., acetonitrile, methanol, ethanol, water, and phosphate buffer at a given pH), different flow modes (isocratic and gradient), different columns (C18 and C8), and different temperature settings were utilised. Acetonitrile and 0.1N phosphate buffer appeared to be an appropriate mobile phase for the separation of Gliclazide and Sitagliptin based on the results of the initial tests, which showed reduced tailing, a shorter retention period, and a superior peak shape [19].

5.1. Optimisation of methods through experimental design

The current study employed a central composite design with 20 trials to examine the impact of three independent variables on seven dependent variables. Three independent variables are flow rate (X1), temperature (X3), and mobile phase % (X2). The dependent variables in this study are the following: SIT (Sitagliptin retention time), gliclazide retention time, resolution factor, SIT (Gliclazide theoretical plate count), Y6 (Sitagliptin tailing factor), and Y7 (Gliclazide tailing factor). The current study employed a central composite design with 20 trials to examine the impact of three independent variables on seven dependent variables. Three independent variables are flow rate (X1), temperature (X3), and mobile phase % (X2). The dependent variables in this study are the following: SIT (Sitagliptin retention time), gliclazide retention time, resolution factor, SIT (Gliclazide theoretical plate count), Y6 (Sitagliptin tailing factor), and Y7 (Gliclazide tailing factor).

5.2. Respondents' model-fitting

To find the best-fit mathematical model, the Design-Expert programme fitted the observed responses from all 20 runs to each model. The models' CVs, R2s (both adjusted and projected), SDs, correlation coefficients, and PRESSs (predicted residual sums of squares) are displayed in Table 3. After considering all replies, the model with the highest R2 value, the lowest SD, CV, and PRESS, and the strongest correlation between adjusted and

expected R2 values was selected. All seven replies (Y1–Y7) were best fit by the quadratic model.

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

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A:FR	B:MP (Organic phase)	C: Temp	RT1	RT2	RS	NTP1	TF2
		ml/min	%	0 C	min	min	num	num	num
1	9	0.27	25	27	1.396	1.696	3.9	6924	1.31
2	15	0.33	25	27	1.261	1.516	3.9	6291	1.1
3	10	0.27	35	27	1.439	1.79	4.5	5711	1.1
4	18	0.33	35	27	1.279	1.615	4.5	6360	1.1
5	8	0.27	25	33	1.338	1.605	4	6997	1.3
6	4	0.33	25	33	1.153	1.428	3.8	5412	1.1
7	12	0.27	35	33	1.364	1.688	4.7	6024	1.3
8	20	0.33	35	33	1.171	1.53	4.5	5914	1.3
9	6	0.249546	30	30	1.45	1.759	4.2	6890	1.4
10	14	0.350454	30	30	1.157	1.453	4	6303	1.2
11	7	0.3	21.591	30	1.282	1.523	3.7	6557	0.99
12	16	0.3	38.409	30	1.319	1.681	4.8	6000	0.98
13	2	0.3	30	24.9546	1.361	1.697	4.3	5957	1.27
14	11	0.3	30	35.0454	1.225	1.514	4.4	5571	1.4
15	19	0.3	30	30	1.29	1.609	4.1	6224	1.2
16	17	0.3	30	30	1.31	1.619	4.1	6290	1.22
17	3	0.3	30	30	1.29	1.6	4.1	6209	1.2
18	1	0.3	30	30	1.304	1.608	4.2	6224	1.24
19	5	0.3	30	30	1.301	1.606	4.1	6291	1.22
20	13	0.3	30	30	1.301	1.617	4.1	6257	1.24

5.2.1. Impact of independent variables on Sitagliptin retention time (Y1):

Following is a quadratic equation that describes the relationship between the independent variables and the Sitagliptin (Y1) retention time.

Sitagliptin (Y1) retention time = 1.30 -0.0854X1 +0.0122X2 -0.0423X3 -0.0041X1X2 - 0.0104X1X3 - 0.0021X2X3

Retention period for sitagliptin is inversely related to both flow rate (X1) and temperature (X3), as seen in the equation. This points to the fact that higher temperatures and flow rates result in shorter retention times. The high coefficient value of X3 indicates that, out of the three variables, temperature has the most impact on the retention time of sitagliptin. The combined interaction terms X1X2, X1X3, and X2X3 have a negative effect on the retention time of sitagliptin. Table 4 displays the results of the analysis of variance (ANOVA) for the data on Sitagliptin retention time. Indicative of statistical significance, the model has an F-value of 499.12. The likelihood of chance playing a role in producing an F-value this high is 0.01%. A p-value of less than 0.0500 indicates that the model term is statistically significant. The model terms X1, X2, X3, and X2X3 hold significant weight in this context. The projected R2 value of 0.9905 and the revised R2 value of 0.9937 are quite close to each other. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. The ratio of 74.640 indicates a strong enough signal. It is possible to navigate the design space with this model. Figs. 3 and 2 show the 3D response surface and 2D contour plots, respectively, showing the effect of independent variables on the sitagliptin retention time (Y1). A and B. As the temperature and flow rate increased, the retention period decreased, according to the plots.

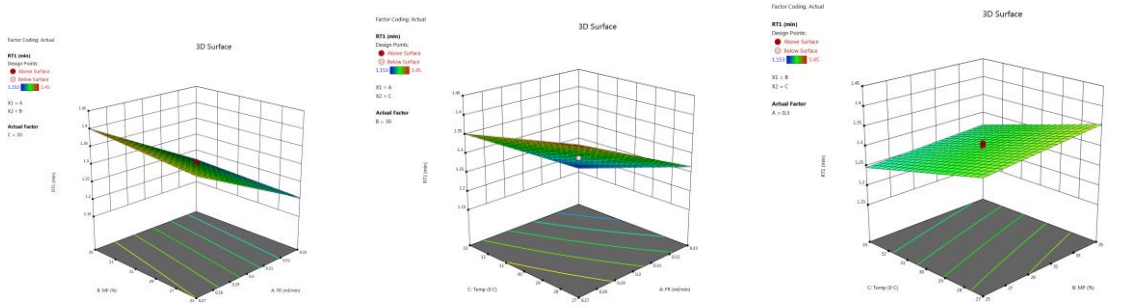
Table 3: Fitting to different polynomial replies for answers Y1 through Y7 using regression analysis.

model	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	Remarks
Y1						
Linear	0.0100	0.9876	0.9852	0.9787	0.0027	Suggested
2FI	0.0065	0.9957	0.9937	0.9905	0.0012	
Quadratic	0.0066	0.9966	0.9935	0.9889	0.0014	
Cubic	0.0073	0.9975	0.9921	0.9892	0.0014	
Y2						
Linear	0.0072	0.9951	0.9942	0.9921	0.0013	Suggested
2FI	0.0074	0.9958	0.9939	0.9878	0.0021	
Quadratic	0.0081	0.9961	0.9927	0.9796	0.0035	
Cubic	0.0072	0.9982	0.9942	0.9208	0.0135	

Y3						
Linear	0.0968	0.9091	0.8921	0.8534	0.2418	Suggested
2FI	0.0980	0.9243	0.8893	0.8548	0.2395	
Quadratic	0.0308	0.9943	0.9891	0.9875	0.0206	
Cubic	0.0389	0.9945	0.9825	0.8914	0.1796	
Y4						
Linear	363.76	0.3595	0.2395	-0.1845	3.894E+06	Suggested
2FI	237.44	0.7771	0.5794	0.5794	0.383E+06	
Quadratic	44.37	0.9940	0.9642	0.9642	1.177E+05	
Cubic	32.92	0.9980	0.9877	0.9877	40574.69	
Y5						
Linear	55.75	0.6545	0.5897	0.3542	92959.95	Suggested
2FI	8.44	0.9936	0.9906	0.9806	2787.08	
Quadratic	8.93	0.9945	0.9895	0.9721	4147.59	
Cubic	7.34	0.9978	0.9929	0.9791	3013.84	
Y6						
Linear	0.1381	0.0285	-0.1536	-0.7588	0.5528	Suggested
2FI	0.1397	0.1922	-0.1806	-0.5064	0.4735	
Quadratic	0.0175	0.9903	0.9815	0.9543	0.0144	
Cubic	0.0177	0.9940	0.9810	0.7935	0.0649	
Y7						
Linear	0.1101	0.2594	0.1205	-0.3458	0.3527	Suggested
2FI	0.1081	0.4198	0.1520	-0.0605	0.2889	
Quadratic	0.0150	0.9914	0.9837	0.9723	0.0073	
Cubic	0.0167	0.9936	0.9798	0.9342	0.0173	

Y1: Sitagliptin retention time Y2: Gliclazide retention time Y3: Resolution factor Y4: Gliclazide number of theoretical plates Y5: Sitagliptin number of theoretical plates Y6: Sitagliptin tailing factor Y7: Gliclazide tailing factor

(A)



(B)

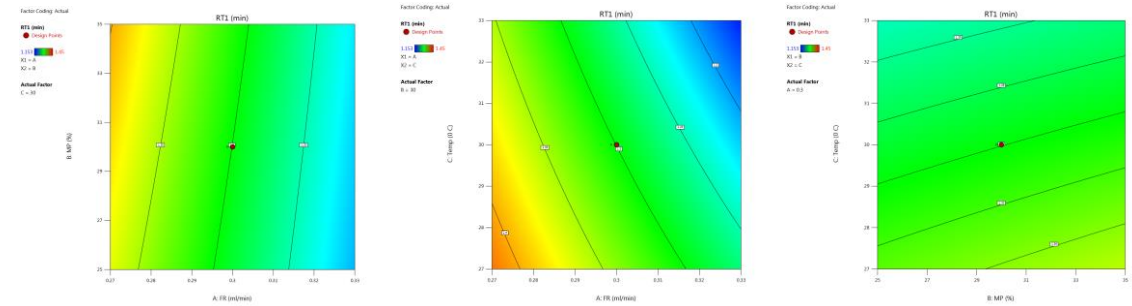


Fig. 2 The three-dimensional response surface plots (A) and two-dimensional contour plots (B) that show how different independent variables affect the Sitagliptin retention time (Y1)

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

	Y1		Y2		Y3		Y4		Y5		Y6		Y7	
	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value
Model	499.12	<0.0001	1077.44	<0.0001	192.30	<0.0001	184.46	<0.0001	334.44	< 0.0001	112.87	< 0.0001	128.21	<0.0001
X_1	2345.95	<0.0001	2022.22	<0.0001	41.90	<0.0001	264.44	<0.0001	1239.52	< 0.0001	13.46	0.0043	181.16	<0.0001
X_2	48.27	<0.0001	577.46	<0.0001	1530.14	<0.0001	242.22	<0.0001	46.51	< 0.0001	2.73	0.1297	0.2339	0.6391
X_3	576.15	<0.0001	632.62	<0.0001	10.47	0.0089	93.83	<0.0001	35.82	< 0.0001	13.10	0.0047	120.47	<0.0001
X_1X_2	3.21	0.0965	-	-	0.0000	1.0000	482.68	<0.0001	113.15	< 0.0001	123.42	< 0.0001	93.32	<0.0001
X_1X_3	20.30	0.0006	-	-	21.11	0.0010	185.90	<0.0001	1.80	0.2032	14.73	0.0033	0.0555	0.8185
X_2X_3	0.8516	0.3729	-	-	5.28	0.0445	28.76	0.0003	569.82	< 0.0001	29.74	0.0003	93.32	<0.0001
X_1^2	-	-	-	-	1.27	0.2858	107.88	<0.0001	-	-	65.60	< 0.0001	47.74	<0.0001
X_2^2	-	-	-	-	29.29	0.0003	0.5879	0.4610	-	-	340.45	< 0.0001	452.23	<0.0001
X_3^2	-	-	-	-	95.50	<0.0001	218.97	<0.0001	-	-	336.90	< 0.0001	100.81	<0.0001
Adequate precision	74.6395		113.9376		50.3509		48.0508		78.9271		42.1740		40.3617	

The independent variables' levels are coded by X1, X2, and X3. The interaction terms are X1X2, X1X3, and X2X3. The quadratic terms are X12, X22, and X32.

5.2.2. Impact of explanatory factors on half-life of Gliclazide (Y2):

The following linear equation can be used to explain how the independent variables affect the retention duration of Gliclazide (Y2).

Y2 Retention time of Gliclazide = 1.61 -0.0882X1 +0.0471X2 -0.0493X3

The equation states that temperature (X3) and flow rate (X1) have a negative effect on the Gliclazide retention time. As both temperature and flow rate rise, Gliclazide's retention period decreases. X1, with its high value, shows that flow rate, rather than temperature or mobile phase %, is the most important factor influencing Gliclazide retention time. The results of the analysis of variance (ANOVA) for the retention time data of gliclazide are shown in Table 4. An F-value of 1076.44 indicates that the model is relevant. The adjusted R2 value of 0.9942 and the predicted R2 value of 0.9921 are quite close to each other, with a discrepancy of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. The ratio of 113.938 indicates that there is a sufficient signal. It is possible to navigate the design space with this model. It is possible to navigate the design space with this model. Figures 3A and B show 2D contour plots and 3D response surface plots, respectively, that show how different independent variables affect the Gliclazide retention time. The retention period decreased with decreasing temperature and flow rate, as shown in the graphs.

(A) (B)

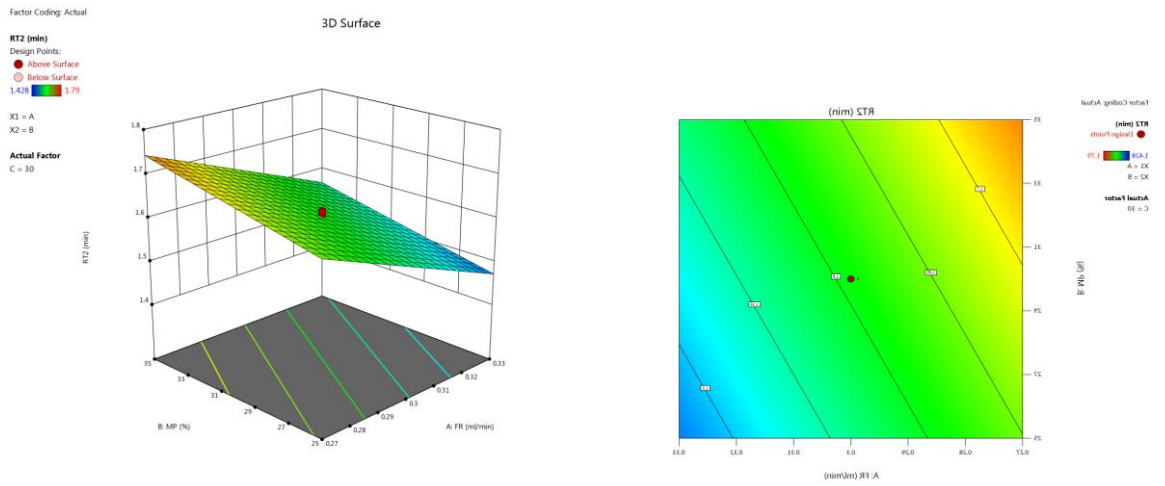


Fig. 3. The effects of independent variables on the retention duration of Gliclazide (Y2) are shown in the 3D response surface plots (A) and related 2D contour plots (B).

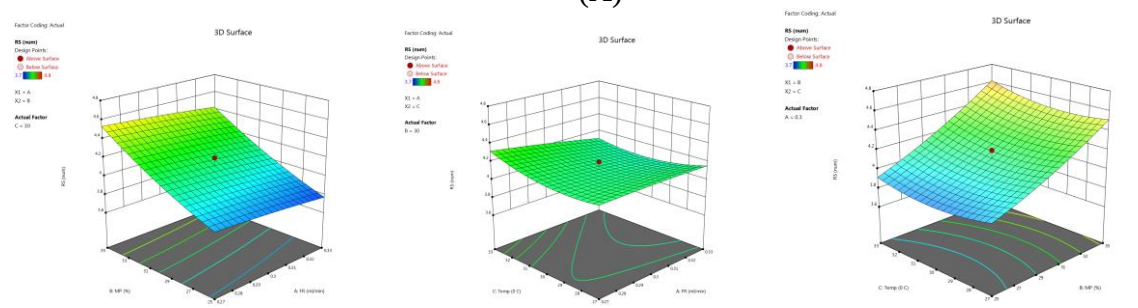
5.2.3. The resolution factor (Y3) is impacted by independent factors:

Here is a quadratic equation that explains the effect of independent variables on the resolution factor (Y3).

Y3 (Resolution Factor) = 4.12 -0.0539X1 +0.3258X2 +0.0270X3 +0.0000X1X2 -0.0500X1X3 +0.0250X2X3 -0.0091X12 +0.0439X22 +0.0792X32

As we can see from the equation, both temperature (X3) and the proportion of mobile phase (X2) have a negative effect on the resolution factor. This means that the resolution factor decreases as the mobile phase percentage and temperature increase. The high coefficient value of X2 indicates that the percentage mobile phase has a greater impact on the resolution factor than temperature and flow velocity. While X1X3 has a negative effect on the resolution factor, the combined interaction term of X1X2 and X2X3 has a favourable impact. The resolution factor data ANOVA results are shown in Table 4. An F-value of 192.30 indicates that the model is statistically significant. A significant model term is one with a p-value below 0.050. These model terms are important: X1, X2, X3, X1X3, X12, and X22. A p-value of less than 0.0500 indicates that the model terms are statistically significant. A reasonably close match exists between the 0.9891 Adjusted R2 and the 0.9875 Predicted R2, with a discrepancy of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. The ratio of 50.351 indicates a strong signal. It is possible to navigate the design space with this model. Figures 4A and B show, respectively, a 2D contour plot and a 3D response surface plot, which show how the independent variables affect the resolution factor. The resolution factor between gliclazide and sitagliptin decreased as the temperature, methanol %, and flow rate increased, according to the graphs.

(A)



(B)

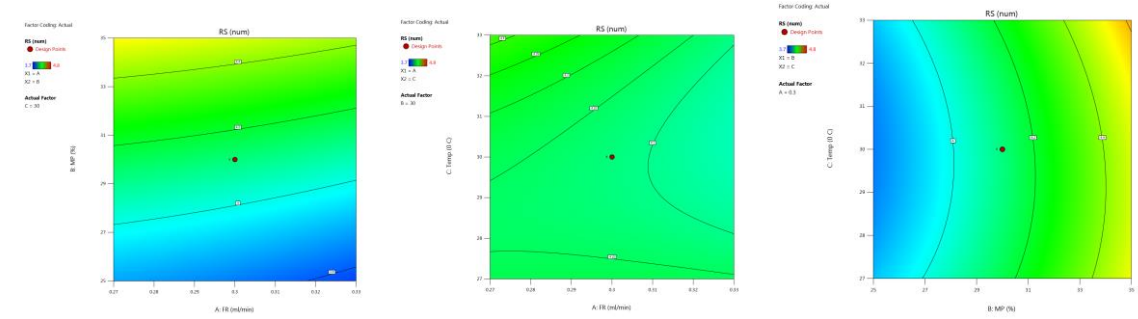


Fig. 4: The correlation between independent variables and the resolution factor (Y3) as shown in the 3D response surface plots (A) and the corresponding 2D contour plots (B).

5.2.4. Effects of explanatory factors on the theoretical plate count of Sitagliptin (Y4):

Independent variables' impacts on the theoretical Sitagliptin (Y4) plate are described by the following quadratic equation.

Y4 theoretical Plates of Sitagliptin = 6249.38 -195.23X1 -186.85X2 -116.29X3 +344.62X1X2 -213.88X1X3 +84.12X2X3 +121.39X12 +8.96X22 -172.94X32

Temperature (X3), flow rate (X1), and mobile phase % all have a negative effect on the theoretical plates of sitagliptin, as shown in the equation. This suggests that the number of theoretical plates of sitagliptin reduces as the flow velocity, percentage of mobile phase, and temperature increase. With a high coefficient value of X1, we can see that flow rate has a greater effect on the sitagliptin theoretical plate than other variables. X1X3 has a negative effect on sitagliptin theoretical plates, although X2X2 and X1X3 have beneficial effects. Table 4 displays the ANOVA findings for the data that was obtained. A substantial model is indicated by an F-value of

184.46. The adjusted R2 value of 0.9886 is in reasonable agreement with the predicted R2 value of 0.9642, with a difference of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. The ratio of 48.051 indicates a sufficient signal. It is possible to navigate the design space with this model. Figure 5A, B shows 2D contour plots and 3D response surface plots that show how the independent variables affect the theoretical plates of sitagliptin. As the flow rate, percentage of mobile phase, and temperature were reduced, the theoretical plates of sitagliptin increased, as shown in the figures.

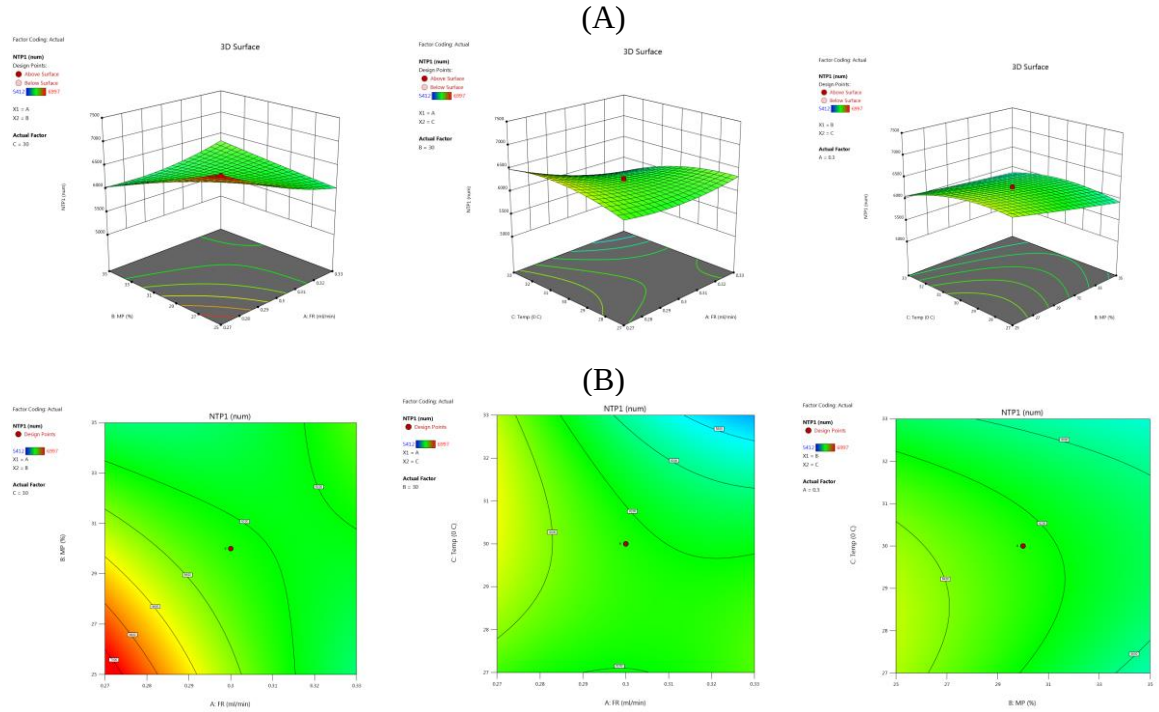


Fig. 5: Plots of the three-dimensional response surface (A) and the corresponding two-dimensional contour (B) illustrating the influence of independent variables on the hypothesised Sitagliptin (Y4) plates

5.2.5. Theoretical plates of Gliclazide (Y5) affected by independent variables:

The following quadratic equation describes the effect of independent factors on the Gliclazide theoretical plate.

Y5 Gliclazide theoretical plates = 9444.35 + 80.43X1 – 15.58X2 – 13.67X3 + 31.75X1X2 + 4.00X1X3 – 71.25X2X3

The equation states that Gliclazide theoretical plates are positively affected by flow rate (X1) and negatively affected by percentage mobile phase (X2) and temperature (X3). As a result, Gliclazide's tailing factor increases with rising temperature and mobile phase percentage but decreases with rising flow rate. On the gliclazide theoretical plates, the cumulative effect of the interaction terms X1X2 and X1X3 is positive. Table 4 shows the results of the analysis of variance (ANOVA) on the collected data. When the F-value is 334.44, it means that the model is considerable. A p-value below 0.05 indicates that the model terms are statistically significant. The adjusted R2 value of 0.9906 is in reasonable agreement with the predicted R2 value of 0.9806, with a discrepancy of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. With a ratio of 78.927, we can see that there is enough signal. It is possible to navigate the design space with this model. Because of this, the quadratic model is an effective method for designing for space. Figure 6A, B show the 3D response surface plots and the associated 2D contour plots, which demonstrate the effect of independent parameters on the Gliclazide theoretical plates. The graphs shown that Gliclazide theoretical plates reduced with rising flow rate but rose with rising temperature and mobile phase %.

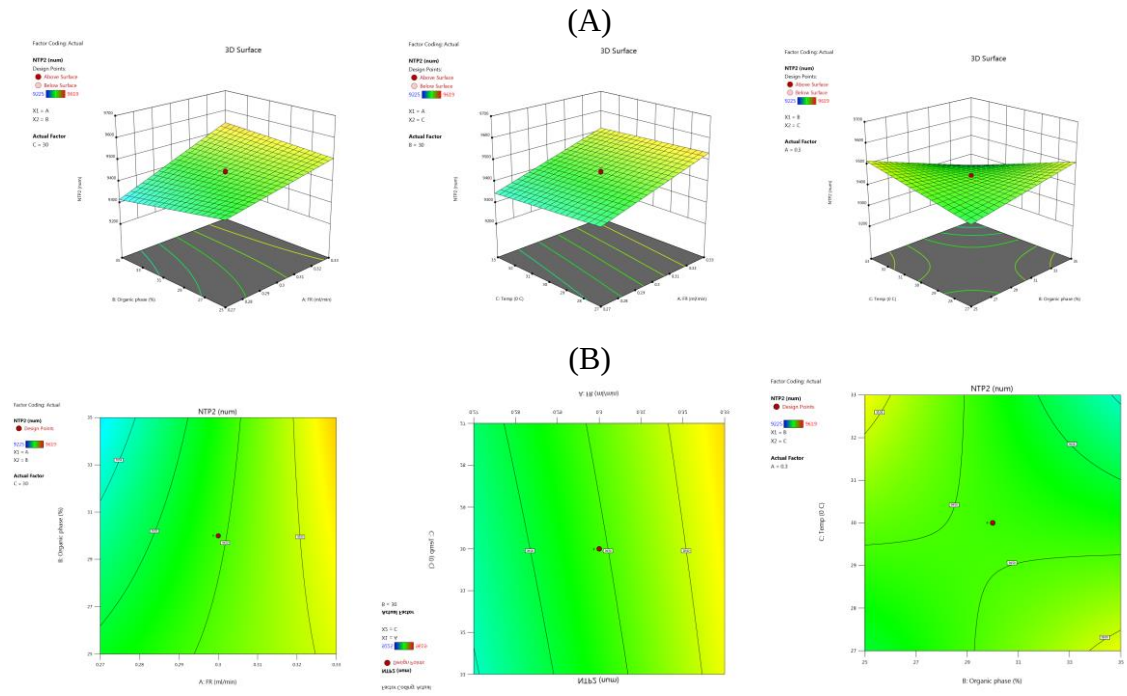


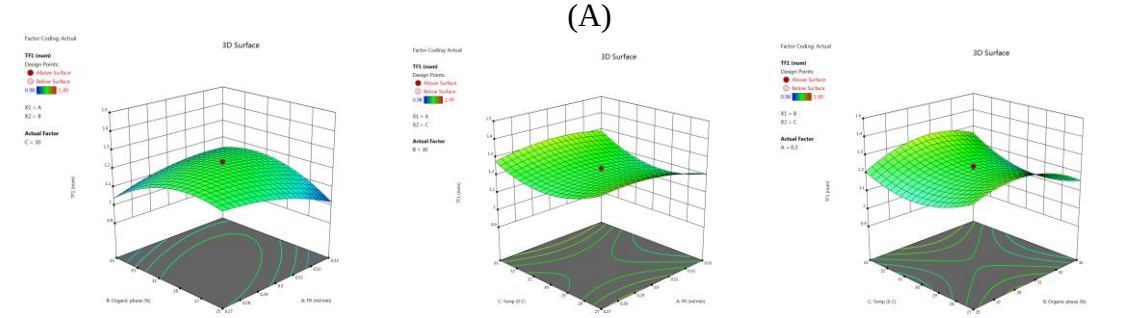
Fig. 6: The 2D contour plots (B) and 3D response surface plots (A) that indicate how the independent variables affect the Gliclazide (Y5) theoretical plates

5.2.6. Aspects of the tailing factor (Y6) of sitagliptin that are affected by independent variables:

To better understand the impact of the independent variables on the sitagliptin tailing factor, consider the following quadratic equation.

Y6 Tailing factor of Sitagliptin = 1.22 – 0.0174X1 – 0.0078X2 + 0.0171X3 + 0.0688X1X2 + 0.0238X1X3 + 0.0338X2X3 – 0.0373X12 – 0.0851X22 + 0.0846X32

The equation gives a positive effect of temperature (X3) on the tailing factor of sitagliptin and a negative effect of flow rate (X1) and percentage mobile phase (X2). This shows that sitagliptin's tailing factor increases with increasing temperature and decreases with decreasing flow rate and mobile phase percentage. When X1X2, X1X3, and X2X3 are combined, they improve sitagliptin's tailing factor. Table 4 shows the results of the analysis of variance (ANOVA) on the collected data. An F-value of 112.87 signifies a statistically significant model. A p-value below 0.05 indicates that the model terms are statistically significant. The adjusted R2 of 0.9815 and the predicted R2 of 0.9543 are in close agreement, with a difference of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. A signal-to-noise ratio of 42.174 is considered adequate. It is possible to navigate the design space with this model. Because of this, the quadratic model is an effective method for designing for space. If we look at Figure 7A, a 3D response surface plot, and Figure 7B, a 2D contour plot, we can see how different parameters influence the tailing factor of sitagliptin. According to the graphs, the tailing factor of Sitagliptin increased with rising temperature and dropped with falling flow rate and mobile phase %.



(B)

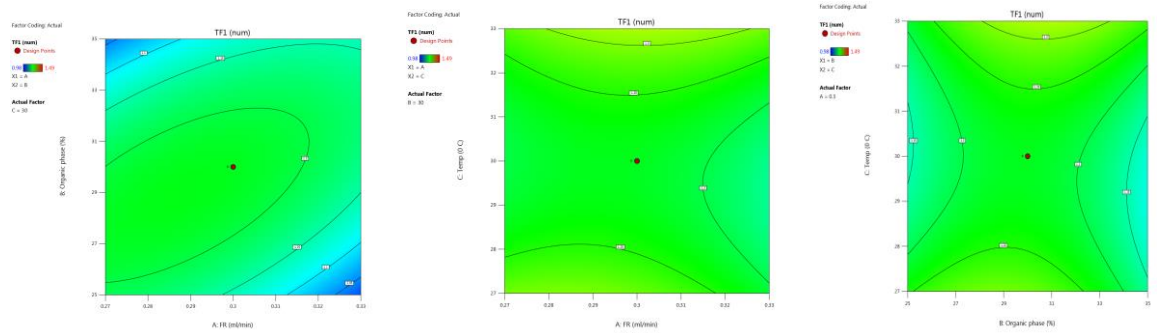


Fig. 7: Plots of the three-dimensional response surface (A) and the corresponding two-dimensional contour (B) illustrating the influence of independent variables on the Sitagliptin tailing factor (Y6)

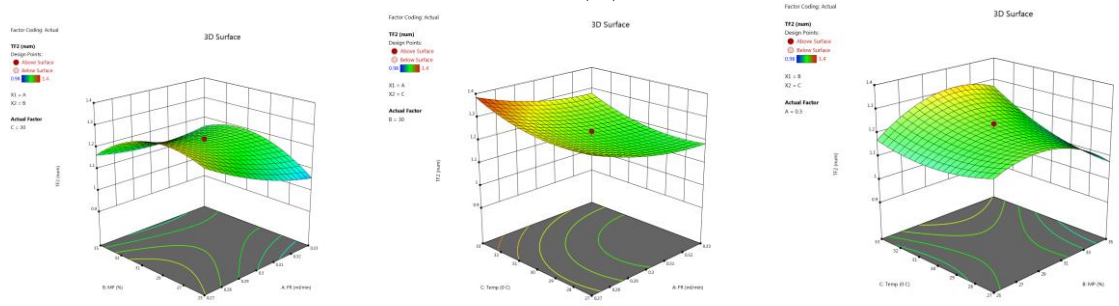
5.2.7. Effects of explanatory factors on Gliclazide's tailing factor (Y7):

To better understand the impact of the independent variables on Gliclazide's tailing factor, consider the following quadratic equation.

Y7 Tailing factor of Gliclazide =1.12 -0.0547X1 -0.0020X2 +0.0446X3 +0.0512X1X2 +0.0012X1X3 +0.0512X2X3 +0.0273X12 -0.0841X22 +0.0397X32

While temperature (X3) positively affects Gliclazide's tailing factor, flow rate (X1) and percentage mobile phase (X2) negatively do, as seen in the equation. Consequently, Gliclazide's tailing factor increases with increasing flow rate and percentage of mobile phase and decreases with increasing temperature. With X1X2, X1X3, and X2X3 as an interaction term, Gliclazide's tailing factor is positively affected. Table 4 shows the results of the analysis of variance (ANOVA) on the collected data. An F-value of 128.21 indicates a significant model. A p-value below 0.05 indicates that the model terms are statistically significant. The adjusted R2 value of 0.9837 and the predicted R2 value of 0.9723 are quite close to each other, with a discrepancy of less than 0.2. There is sufficient accuracy in measuring the signal-to-noise ratio. A ratio greater than four is optimal. A ratio of 40.362 indicates a sufficient signal. It is possible to navigate the design space with this model. This means the quadratic model is an effective method for designing for space travel. Figure 8A and B show the equivalent 2D contour plots and 3D response surface plots, which demonstrate the impact of independent factors on the Gliclazide tailing factor. According to the graphs, the tailing factor of Gliclazide reduced with increasing temperature and flow rate concentrations but increased with increasing mobile phase %.

(A)



(B)

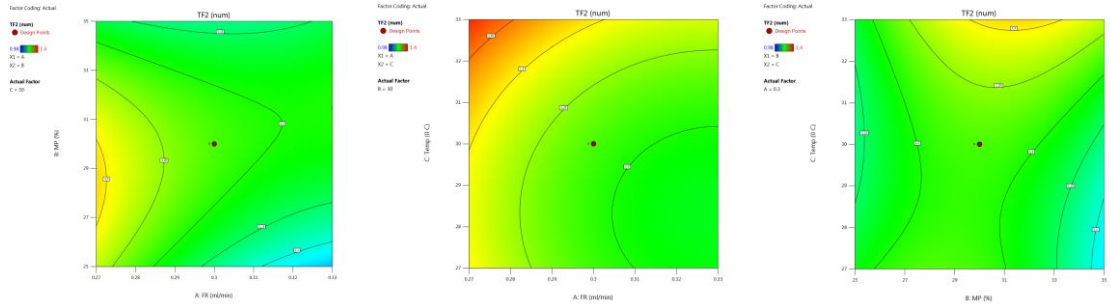


Fig. 8: Plots of the three-dimensional response surface (A) and related two-dimensional contour (B) illustrating the influence of independent factors on the Gliclazide tailing factor (Y7)

5.2.8. Choosing the most suitable chromatographic parameters

To choose the best chromatographic parameters, the Design expert® program's numerical optimisation method was employed. The programming supplied the optimal chromatographic settings for the separation of sitagliptin and gliclazide, which included a mobile phase consisting of 30.6 volumes of acetonitrile and 69.4 volumes of 0.1% OPA, a flow rate of 0.31 mL/min, a column temperature of 30 °C, and UV detection at 248 nm. Under optimum chromatographic conditions, answers were documented following the injection of 60 µg/mL of sitagliptin and 18 µg/mL of gliclazide into the UPLC system. Table 5 displays the anticipated and actual values of the answers as well as the % residuals for each. The QbD design was used to identify the optimal chromatographic conditions for the separation of sitagliptin and gliclazide, and the residual values from 0.320 to 2.53 showed that it was valid. The approach presented was validated using the improved chromatographic conditions. Based on the observed values, the core composite design was validated by the low % residual error values.

Table 5 Optimal chromatographic conditions yielded the following expected and observed values of responses:

Response	Predicted Value	Observed value	Residual values (%)
Retention time of Sitagliptin (min)	0.9937	0.9905	0.0006
Retention time of Gliclazide (min)	0.9942	0.9921	0.0008
Resolution factor	0.9891	0.9875	0.0095
Theoretical plates Sitagliptin	0.9886	0.9642	1968.43
Theoretical plates Gliclazide	0.9906	0.9806	71.27
Tailing factor Sitagliptin	0.9815	0.9543	0.0003
Tailing factor Gliclazide	0.9837	0.9723	0.0023

Table 6 System suitability test (n=6):

Parameter	Sitagliptin	Gliclazide	Acceptance Criteria
Retention time	1.26 ± 0.005	1.56 ± 0.005	—
Peak area	1220035	365865	<1 for n≥5
RSD % of peak area	0.4	0.6	—
Theoretical plates (N)	6172 ± 24	9459 ± 65	<1 for n≥5
Tailing factor (T)	1.14 ± 0.02	1.12 ± 0.005	>2000
Resolution (Rs)	1.269	1.572	≤2.0

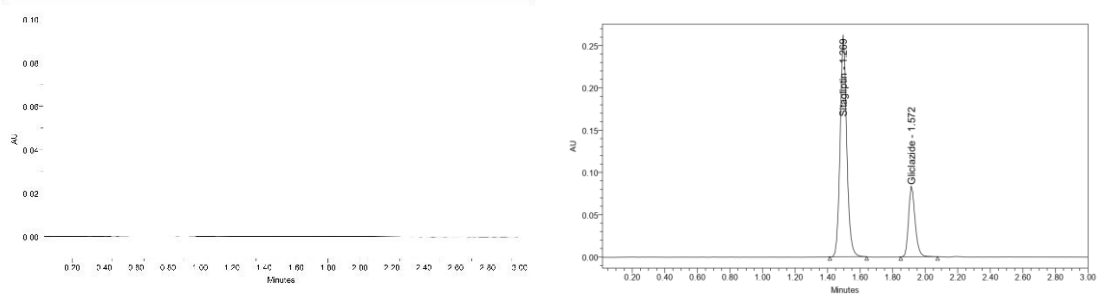


Fig. 9: Chromatograms demonstrating the specificity of the established UPLC method: placebo (A) and

conventional Sitagliptin and Gliclazide (B).

5.2.9. Validation of the technique

The characteristics for system suitability and the matching acceptance criteria are displayed in Table 6. All of the theoretical plate counts, tailing factors, resolution factors, and percentages of relative standard deviations (RSDs) meet the reference values. For Sitagliptin and Gliclazide, the retention time and peak area are less than 1, the theoretical plate counts are 6172 ± 24 and 5436 ± 65, respectively. For Gliclazide, the tailing factor is 1.21, and for Sitagliptin it is 1.15. The resolution factor is 4.0, and for percentage RSD it is 2. The results are displayed in Figure. Figure 9 shows that the optimised chromatogram and standard calibration curves for Gliclazide and Sitagliptin, respectively, were linear within the concentration ranges of 3-18 µg/mL and 10-60 µg/mL. The regression coefficient (r2) for sitagliptin was 0.9999 when the equation for the drug's effect was y = 30432x + 4316.3. It was found that the regression coefficient (r2) was 0.9999 using the regression equation y = 43107X + 2557.2. The LOD and LOQ values for Sitagliptin were determined to be 0.28 µg/mL and 0.86 µg/mL, respectively, whereas the values for Gliclazide were 0.19 µg/mL and 0.56 µg/mL. According to Table 7, the relative standard deviation of the intraday and interday precision for Gliclazide and Sitagliptin was less than or equal to 2. The percentage recovery for sitagliptin was 99.15% to 100.54%, whereas for gliclazide it was 99.93% to 100.58%, as shown in Table 8. Fig. 10 shows how specific the created procedure is. Because the formulation excipients did not co-elute with Gliclazide and Sitagliptin at the retention time of the placebo and blank samples, the specificity of the established method was proven. Table 9 shows the findings of the robustness data. Retention time percentage RSD and peak area RSD were both under 2%, indicating very little variance. System suitability characteristics including theoretical plates, tailing factor, and resolution factor remained unchanged. The commercial formulation contained 99.73 mg of sitagliptin and 100.14 mg of gliclazide as a percentage of drug assay. Slight changes in the peak areas of sitagliptin and gliclazide before and after leaving the solutions at room temperature for 24 hours show that both drugs are stable in solutions.

Table 7 Sitagliptin with gliclazide linearity:

S.no	Conc (µg)	Sitagliptin Peak area	Conc µg)	Gliclazide Peak area
01	0	0	0	0
02	10	306931	3	130329
03	20	618691	6	263855
04	30	917311	9	391089
05	40	1212845	12	517604
06	50	1528894	15	649003

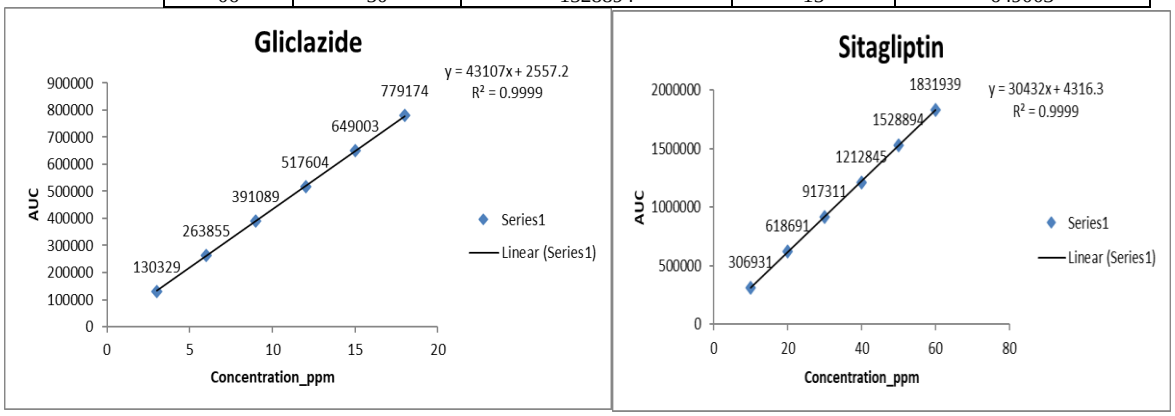


Fig. no. 10 Sitagliptin and Gliclazide calibration curve

Table 8 Recovery studies of Sitagliptin and Gliclazide:

Compound	Contents (µg)	Quantity added (µg)	Recovered amount (µg)	Recovery (%)	%RSD
Sitagliptin	40	20	19.87	99.35	99.87
	40	40	40.19	100.47	0.90
	40	60	60.00	100.00	0.90
Gliclazide	12	06	06	100.00	99.99
	12	12	12	100.00	0.18
	12	18	18	100.00	0.2

Table 9 Intraday and interday precision of Sitagliptin and Gliclazide:

S.no	Sitagliptin		Gliclazide	
	Intra-day Precision	Inter-day Precision	Intra-day Precision	Inter-day Precision
01	1210984	367442	1213577	368934
02	1227845	369463	1214467	367533
03	1219546	368833	1206745	362434
04	1210885	365923	1213467	367304
05	1215457	367073	1216578	367433
06	1227545	368378	1210606	364521
Mean	1218710	367852	1212573	366360
Std.dev	7664.3	1290.8	3442.9	2401.6
% RSD	0.6	0.4	0.3	0.7

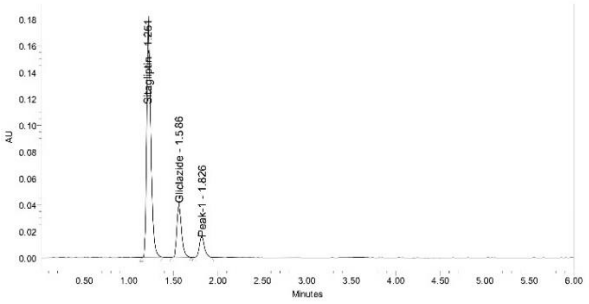
5.2.10. Investigations on forced deterioration

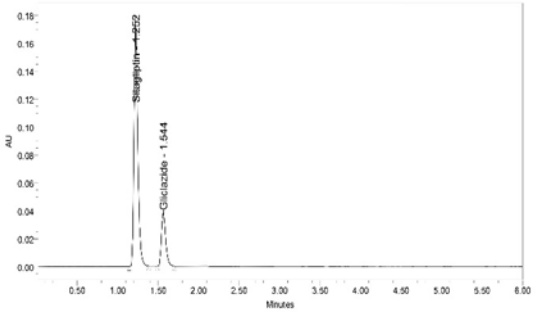
Figure 11 and Table 10 show the results of the Gliclazide and Sitagliptin forced degradation investigations conducted under different stress conditions. Neither sitagliptin nor gliclazide breakdown more than 2% in the acid, alkali, heat, and neutral environments provided. Under oxidative degradation, sitagliptin deteriorated by 1.05% and gliclazide by 1.88%, with a single peak at 1.21 minutes. Two degradation peaks occurred at 1.218 and 1.572 minutes for Sitagliptin and Gliclazide, respectively, and their degradation rates were 0.06 and 1.89% when subjected to photolytic degradation. Furthermore, it was observed that the deterioration peaks were interference-free.

Table 10: Sitagliptin and Gliclazide Stability Studies

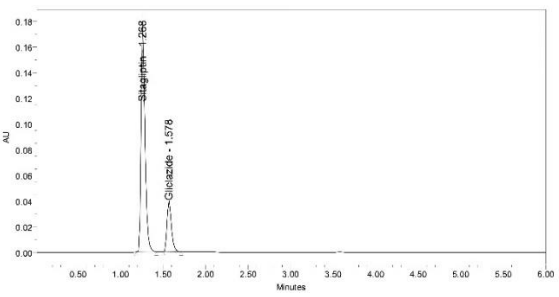
Stress Condition	**Treatment**	**% Degradation**	
		Sitagliptin	**Gliclazide**
Acid hydrolysis	2N HCl, 60 °C for 30 min	5.15	6.78
Alkaline hydrolysis	2N NaOH, 60 °C for 30 min	5.96	6.69
Oxidative degradation	60% H_2O_2 60 °C for 30 min	1.05	1.88
Thermal degradation	105 °C for 6 h	0.30	1.90
Photolytic degradation	UV light for 7 days	0.06	1.89
Neutral hydrolysis	Water at 60 °C for 6 h	0.48	0.98

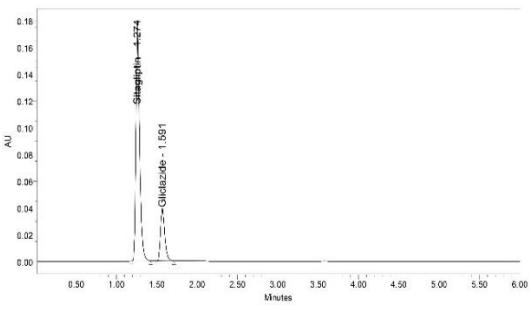
(A) (B)





(C) (D)





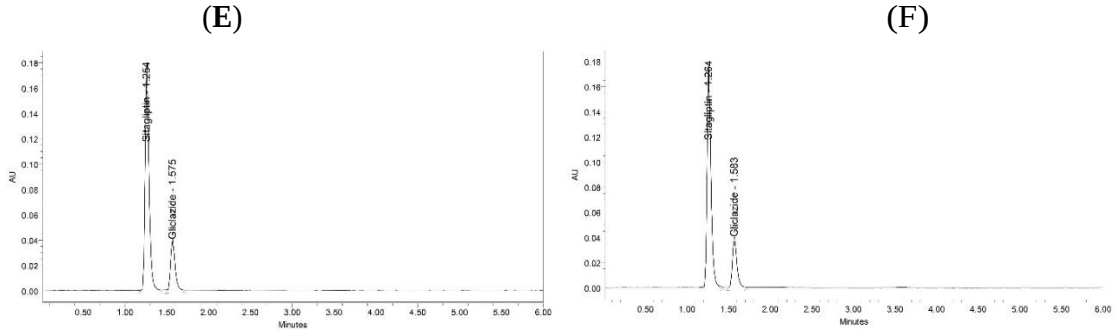


Fig. 11 chromatograms illustrating the sitagliptin and gliclazide degradation profiles in response to various stress conditions, including acidic, alkaline, oxidative, dry heat, neutral, and photolytic degradation.

5.2.11. Subject under consideration

In order to determine the concentrations of Gliclazide and Sitagliptin utilising AQbD, this research established an RP-UPLC method. The chromatographic conditions for the proposed approach were optimised using a central composite design (CCD) with three components over five stages in the Design-expert® software (Stat-Ease Inc., USA). Utilising polynomial equations, 3D response plots, and 2D contour plots, the impact of three independent variables—temperature, flow rate, and methanol percentage—on dependent variables—retention time, resolution factor, number of theoretical plates, and tailing factor—was investigated. The statistical significance of the model and its terms were assessed using ANOVA. Next, a numerical optimisation method was used to forecast the best chromatographic parameters. Polynomial equations and 3D-response plot showed that sitagliptin and gliclazide retention durations were most affected by temperature and flow rate, respectively. A more important element influencing the resolution factor than temperature and flow rate was the proportion of acetonitrile in the mobile phase. Flow rate was shown to have a more substantial effect on the number of theoretical plates for both drugs. In terms of the tailing factor, it was shown that flow rate had a more significant impact on Sitagliptin than % acetonitrile did on Gliclazide. A significant agreement between expected and actual values and a low residual error show that the QbD model selected the optimal chromatographic parameters for gliclazide and sitagliptin estimation.

The purpose of validation is to ensure that analytical procedures are reliable and suitable for their intended usage. The developed RP-UPLC method was confirmed to be in accordance with the requirements of ICH Q2 R1. To make sure the selected chromatographic system is working properly during the analysis, the system suitability test was run, which is an important step in developing the method. Every one of the assessed parameters—theoretical plates, tailing factor, resolution factor, and percentage RSD for peak area and retention duration—fell squarely within the suggested ranges, proving that the chromatographic system was appropriate. There is a strong relationship and excellent linearity for both medications across the proposed concentration ranges, as shown by the correlation coefficient (r2) values > 0.9999. Reduced LOD and LOQ values demonstrated that the proposed method was highly sensitive for calculating pharmaceutical dose forms of sitagliptin and gliclazide. The term "analytical precision" describes the level of agreement between readings taken from different samples under the same conditions. Percentage RSD values for intraday and interday precision assessed at three separate levels were less than 2%, demonstrating the superior precision of the devised approach. How well the experimental result matches the real value is what recovery studies are all about. With recovery percentages ranging from 99.15% to 100.54% for Sitagliptin and from 99.93% to 100.58% for Gliclazide, respectively, there was minimal variance and excellent agreement between the experimental and actual data. Analytical methods are considered specific if they can detect the target analyte in commercially available formulations despite the presence of impurities, degradation agents, and excipients. Unique to the developed method for the measurement of sitagliptin and gliclazide from pharmaceutical dosage forms is the absence of co-eluting interference peaks at the retention times of both medications. Reliability in everyday applications is shown by a method's robustness, which is its capacity to maintain consistency in the face of slight alterations in chromatographic parameters. The method's durability was demonstrated by its low percentage RSD values and the fact that system suitability parameters did not undergo significant changes in response to deliberate changes in experimental circumstances. The current RP-UPLC method can be used to estimate the amounts of sitagliptin and gliclazide in commercial formulations based on the percentage assay findings (ranging from 99.73 to 100.14% for label claim). After one

day of storage at room temperature, the stability study confirmed that the solutions of Gliclazide and Sitagliptin were unchanged.

Stability studies are crucial for determining the efficacy of pharmaceuticals and other therapeutic goods. The method's ability to evaluate the stability of sitagliptin and gliclazide is demonstrated by assessing their deterioration following exposure to different stress scenarios. Neither medicine was unstable in neutral, acidic, or alkaline conditions, but it did not hold up in oxidative or photolytic ones. These results show that the current method is stable.

6. Conclusions

Applying analytical quality by design methodology, particularly central composite design, allowed for the optimisation of sitagliptin and gliclazide quantification using the RP-UPLC method. The optimal chromatographic conditions for the separation of sitagliptin and gliclazide were as follows: 30.6 volumes of acetonitrile, 69.4 volumes of 0.1% OPA, a flow rate of 0.31 mL/min, a column temperature of 30 °C, and UV detection at 248 nm. The method showed clearly defined peaks at 1.269 and 1.572 minutes for sitagliptin and gliclazide, respectively. After the method was validated according to ICH standards, it demonstrated strength, accuracy, precision, sensitivity, and specificity. In addition to degradation peaks, well-resolved peaks for Sitagliptin and Gliclazide were seen in the forced degradation tests. This study's findings support the use of AQbD as a method optimisation tool and confirm that the existing approach is suitable for accurately calculating the dose forms of sitagliptin and gliclazide in pharmaceuticals.

Reference

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