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AQBD ENABLED OPTIMIZATION OF RP-HPLC METHOD FOR THE ESTIMATION OF PIOGLITAZONE AND VILDAGLIPTIN AND ITS FORCED DEGRADATION STUDIES

P V Devi Swapna, G. Saravanan*

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ABSTRACT

Background: Diabetes mellitus is a metabolic disorder that is managed with combination therapy. Pioglitazone improves insulin sensitivity by activating PPAR-γ, whereas vildagliptin increases incretin activity by inhibiting DPP-4. Their fixed-dose combination requires reliable analytical methods for quality control and stability assessment. This study aimed to develop a green, stability-indicating RP-HPLC method using the Analytical Quality by Design (AQbD) approach. **Methodology:** A systematic AQbD strategy employing Central Composite Design (CCD) with 13 experimental runs was used to optimize critical analytical parameters. Chromatographic separation was performed on an Agilent 1260 Infinity II HPLC system using a Phenomenex C18 column (150 × 4.6 mm, 5 µm). Detection was carried out at 220 nm with a flow rate of 0.8 mL/min. The method was validated in accordance with ICH guidelines, and greenness was evaluated using the AGREE, MoGAPI, and BAGI metrics. **Results and Discussion:** Vildagliptin and pioglitazone were well resolved with retention times of 2.21 min and 3.47 min, respectively. Validation results demonstrated excellent precision, accuracy, and reliability. The method exhibited acceptable greenness with a high BAGI score of 82.5, an AGREE score of 0.66, and a MoGAPI value of 74. Stress degradation studies confirmed its stability-indicating capability, with maximum degradation under alkaline and oxidative conditions, while acidic, thermal, and photolytic stresses showed minimal effects. **Conclusion:** The proposed AQbD-driven, eco-friendly RP-HPLC method is sensitive, stability-indicating, and regulatory-compliant, offering reduced analysis time, enhanced robustness, and improved environmental performance, making it a sustainable and efficient approach for routine estimation of pioglitazone and vildagliptin.

INTRODUCTION

"Pharmaceutical Quality by Design (QbD), a systematic developmental approach that begins with specific objectives and focuses on comprehending and managing both products and processes through sound scientific principles and quality risk management" [1]. The Analytical Quality by Design (AQbD) strategy in RP-HPLC represents a contemporary, structured method development approach that prioritizes creating robust,

*Department of Pharmacy, Karpagam Academy of Higher Education, Coimbatore-641032, Tamil Nadu, India.

***For Correspondence:** **drsaravanan.g@kahedu.edu.in**

dependable, and effective chromatographic methods by thoroughly understanding the method's variables and how they interact [2].

Initially, formulation scientists used the Quality by Design (QbD) technique to ensure product quality throughout development. Furthermore, the demand for highly precise and accurate analytical methods in industry led to the investigation of their application in analytical method development, which emerged as the AQbD methodology [3]. These concerns have been taken seriously by several pharmaceutical regulatory bodies, including the US FDA and ICH, which have developed industry standards to address them. ICH recently introduced ICH Q14 recommendations for the creation of analytical techniques and the revision of Q2 (R1) and Q2 (R2) as part of this endeavor. Additionally, these organizations promote the development of analytical procedures by utilizing Analytical Quality by Design (AQbD) methodology [4]. Developing a robust RPHPLC method for any drug relies primarily on the analytical quality-by-design strategy. This involves finding the Analytical Target Profile (ATP's) first. Next, determine the Critical Method Parameters that affect the Critical Quality Attributes. The risk assessment is investigated to determine how the CQA affects the CMP by means of Ishikwava Fishbone Diagram. This evaluation guides the development of a sound analytical technique and helps identify the elements that most significantly affect quality [5]. Based on a preliminary risk assessment and chromatographic knowledge, buffer pH and buffer concentration were selected as critical method parameters (CMPs) for optimization using Central Composite Design (CCD). Since both pioglitazone and vildagliptin contain ionizable functional groups, their retention, selectivity, and peak symmetry are strongly influenced by mobile-phase pH. Buffer concentration affects ionic strength, which in turn impacts resolution and column efficiency. Other variables, such as flow rate and temperature, showed comparatively lower risk ranking and predictable effects within the studied range. Therefore, buffer pH and buffer concentration were prioritized for systematic multivariate optimization to ensure robust and reliable method performance within the defined design space.

The regulatory authority of India, the Central Drugs Standard Control Organization, has authorized a fixed-dose combination (FDC) of pioglitazone (PIO) and vildagliptin (VIL). The combination has significant regulatory approval, documented clinical efficacy, and an acceptable safety profile, making it a viable alternative to current therapies for type 2 diabetes mellitus.

Green Analytical Chemistry (GAC) and White Analytical Chemistry (WAC) principles aim to develop and implement analytical methods that minimize environmental impact, improve safety, and promote sustainability. Several measures, including AGREE, MoGAPI, and BAGI, have been developed to assess the greenness of analytical methods efficiently [6]. The 12 principles of Green Analytical Chemistry form the basis for a comprehensive tool known as AGREE (Analytical Greenness Metric) [7]. It evaluates several factors, including energy consumption, waste, toxicity, automation, direct analysis, sample size, integration, and operator safety [8].

The result is an easily comprehensible circular pictogram with colored segments signifying adherence to each principle and a single, centrally located number score ranging from 0 to 1 that rapidly conveys total greenness. MoGAPI (Modular Green Analytical Procedure Index) builds on GAPI by splitting the analytical procedure into individual modules for independent evaluation [9]. Each module, including sample collection, preparation, reagent usage, and disposal, is assigned a green, yellow, or red grade. The modular approach provides precise information and helps identify individual process stages that may be altered to improve environmental performance [10]. BAGI (Blue application Grade Index) considers not only the greenness but also the practical application ("whiteness") of an analytical procedure.

It considers other criteria such as throughput, automation, sample handling, resource selection, and appropriateness for routine use. BAGI is particularly helpful in finding approaches that are both green and successful for real-world applications [11]. Combining AGREE, MoGAPI, and BAGI for evaluating analytical techniques alters method rankings by providing a comprehensive, balanced assessment that accounts for environmental effects, thorough procedural step analysis, and practical application. This method is more closely aligned with contemporary trends in "White Analytical Chemistry," which prioritize sustainability, efficacy, and usefulness. Vildagliptin, a frequently used DPP-4 inhibitor, demonstrated considerable glucose-lowering efficacy as both an initial medication and an add-on treatment in multiple clinical studies in patients with

T2DM. It effectively reduces the mean hyperglycemic amplitude by reducing oxidative stress and inflammation [12]. Vildagliptin inhibits DPP-4, thereby preventing the breakdown of GLP-1; elevated GLP-1 levels improve glucose control. Its IUPAC name is 2S)-1-[(3-hydroxy-1-adamantyl)amino]acetyl] pyrrolidine-2-carbonitrile [13]. Pioglitazone, an oral antidiabetic drug from the thiazolidinedione (TZD) family, is a potent, well-tolerated insulin sensitizer with multiple benefits in managing type 2 diabetes and associated metabolic disturbances. The drug acts on PPAR gamma 1 and PPAR gamma 2 to increase insulin sensitivity, thereby improving glycemic control in individuals with Type 2 diabetes [14]. It has the IUPAC name 5-{[4-[2-(5-ethylpyridin-2-yl) ethoxy] benzyl] methyl} thiazolidine -2, 4-dione [15]. The structures of pioglitazone and vildagliptin are shown in Figures 1a and 1 b.

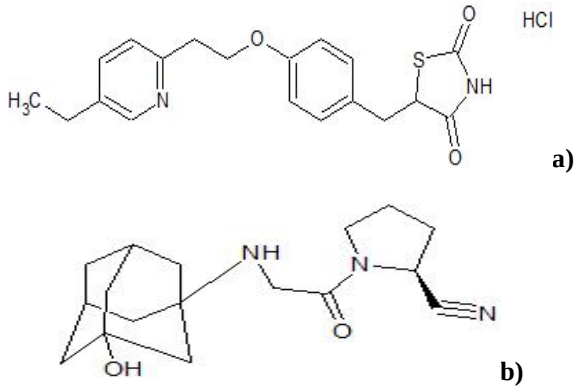


Figure 1: Structure of a) Pioglitazone and b) Vildagliptin

Based on the literature search, a few analytical methods, such as UV [16], HPTLC [17], and RP-HPLC [18], were reported for the estimation of pioglitazone and vildagliptin in formulations. There is very little in-depth research focused specifically on the two-drug combination. The majority of available techniques for these pharmaceuticals still rely on traditional methods or altering one aspect at a time, rather than adopting organized approaches such as design of experiments (DoE), risk assessment, mapping the design space, or defining key quality characteristics (CQA). Hence, the proposed work relies on the principles of AQbD to develop a novel, green RPHPLC method for estimating the drugs and to apply the method to their forced degradation behavior. Stress degradation studies play a vital role in validating an RP-HPLC method for the simultaneous estimation of pioglitazone and vildagliptin. They ensure that the method can clearly separate and quantify the active drugs from any breakdown products, even under harsh conditions such as acids, bases, oxidizing agents, heat, or light.

MATERIALS AND METHODS

The reference standards of pioglitazone and vildagliptin were procured from Yarrow Chem Products, Mumbai, India, and were of analytical grade with a declared purity of ≥99%. The marketed tablet with brand name PIOZ-V, Batch No. 48018924, Exp-11/2025, Mfg by USV PVT was procured from Netmeds online. Acetonitrile and water from Qualigen, Thermo Fisher Scientific India Pvt. Ltd., and Merck Life Sciences Pvt. Ltd.

Framework for AQbD method development

1. **Identifying Analytical Target Profile (ATP), Critical Quality Attributes (CQA), Critical Method Parameter (CMP), and Critical Method Material Attributes (CMMA)**

ATP includes the method type (RP-HPLC), the sample, and the method purpose (drug assay), among other details. Critical Analytical Attributes (CAA) include Retention time, Theoretical plates, Asymmetry, and Resolution. Critical Method Parameters (CMP) are Mobile Phase Ratio, Buffer pH, Buffer Strength, Flow rate, and Injection volume. Critical Method Material Attributes (CMMA) includes Purity of reagents, Stationary phase, glassware types, etc. The probable effect of CMPs on CAAs before method development trials was estimated and outlined.

2. **Risk assessment**

It identifies possible risks in a method, that is, identifying the critical method parameters - mobile phase composition, pH, column temperature, and detector wavelength that affect the method performance like retention time, resolution, peak shape, and theoretical plates owing to slight changes in factors or challenges such as various laboratories, analysts, equipment, reagents, and days [19], [20]. Critical aspects for experimental design and optimization (DoE) are prioritized based on the risk assessment to create a robust approach that maintains its dependability under usual changes [21] as shown in Table 1 and Figure 1.

3. **Optimization and Experimental Design**

This was done by using Design Expert Stat-Ease software version 13.0.5.0. The impacts of pH, flow rate, and organic phase composition on the crucial quality parameters of retention time, resolution, and theoretical plates were thoroughly assessed using Central Composite Design (CCD). Two factors and three responses made up CCD, yielding 13 experimental runs. The experimental trials are illustrated in Table 2. The Buffer pH (Factor A) and buffer concentration (Factor B) were examined as crucial factors in the Central Composite Design. The pH was

adjusted between 3.00 (Coded low) and 7.00 (coded high). Buffer concentration varied from 57.93 to 70.00 % (coded low: 60.00, coded high: 70.00). Shown in Table 3. Based on preliminary research and existing literature, these ranges were selected to enable a comprehensive investigation of the design space relevant to chromatographic performance. ANOVA and regression statistics showed that the produced response surface models indicated substantial individual and interaction effects, enabling the determination of appropriate chromatographic settings. The coding made response surface modeling and effective statistical analysis of the data easier. To confirm method performance in accordance with ICH Q2 (R1) criteria, the optimal conditions identified by CCD were further tested.

Table 1: Summary of Final Risk Assessment

CMPs	CAAs			
	Retention Time	Theoretical Plates	Asymmetry	Resolution
Buffer strength	Low	Low	Low	Low
Buffer pH	High	High	High	High
Mobile Phase Ratio	High	High	High	High
Flow rate	Low	Low	Low	Low
Wavelength	Low	Low	Low	Low
ColumnTemperature	Low	Low	Low	Low
Injection volume	Low	Low	Low	Low

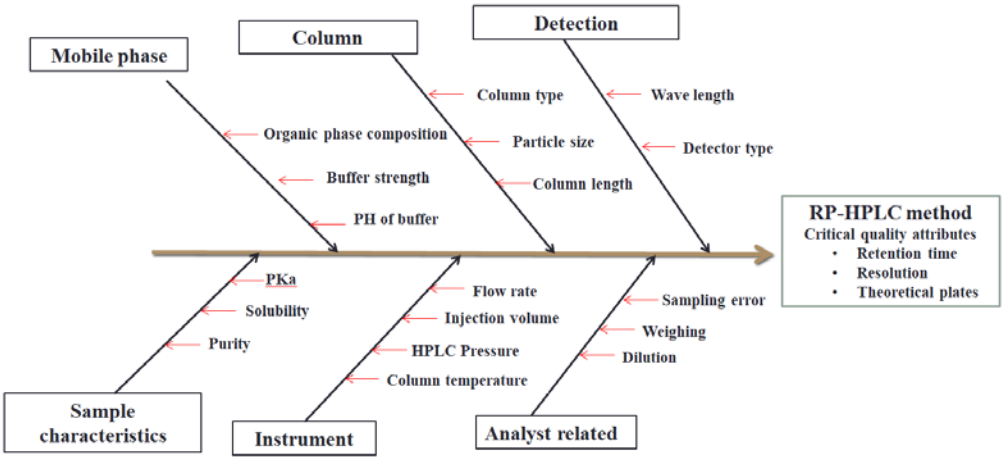


Figure 2: Ishikawa diagram

Table 2: Design trials obtained using CCD Design

		Factors		Responses		
Std	Run	A: Buffer pH	B: Buffer Conc	Retention Time of Vil	Resolution	Theoretical Plates
10	1	5	70	1.85	45.15	4328
11	2	7	65	2.3	15.95	4652
12	3	7	70	2.34	25.94	4689
8	4	3	60	1.62	1a6.28	5163
3	5	3	70	1.65	27	4178
4	6	5	60	1.76	35.04	4698
5	7	7	60	2.21	8.41	4587
1	8	3	65	1.66	20.35	4689
13	9	5	65	1.83	39.61	4587
7	10	7	60	2.2	9.42	4687
9	11	7	60	2.21	9.45	4699
6	12	7	57.9289	2.13	9.41	4654
2	13	7	60	2.21	9.34	4689

Table 3: Factors coded lower limit and higher limit

Factor	Name	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.	%RSD
A	Buffer pH	3.00	7.00	3.00	7.00	5.62	1.71	30.42
B	Buffer concentration	57.93	70.00	60.00	70.00	63.30	4.45	7.03

Instrument and Chromatographic Conditions

HPLC (Agilent Technologies 1260 Infinity II with 1260 Quaternary Pump VL G7111A) with Phenomenex Kinetex XB-C-18 (150 x 4.6 mm, 5 µm) is employed for optimization at 30 °C. Mobile phase selected includes 5 mM Disodium Hydrogen Phosphate Buffer, pH 7, adjusted with orthophosphoric acid, and Acetonitrile (60:40 % v/v) at a wavelength of 220nm and a flow rate of 0.8 mL/min.

Preparation of Standard stock solution

Stock solutions for vildagliptin and pioglitazone were prepared separately by accurately weighing each drug into separate 10ml and 100ml volumetric flasks. To this, diluent was added, and the solution was made up to a final concentration of 500 µg/ml vildagliptin and 1000 µg/ml pioglitazone. The pioglitazone solution is subsequently diluted to 100 µg/ml.

Preparation of the mixture working standard

In a 10 ml volumetric flask, 1.0 ml of Vildagliptin and 1.5 ml of pioglitazone stock solutions were transferred, diluted to volume with the diluent, and mixed thoroughly.

Preparation of Drug Product Sample Solution

10 marketed tablets (PIOZ-V, Batch No. 48018924, Exp – 11/2025, Mfg by – USV PVT. LTD.) were weighed, and the average weight was calculated. The tablets were crushed and powdered. Tablet powder containing 5 mg Vildagliptin and 1.5 mg Pioglitazone was accurately weighed and transferred to a 10 ml volumetric flask, made up to the mark with diluent, sonicated for ten minutes with intermittent shaking, filtered through a 0.45 micron nylon syringe filter, and 1 ml of this filtrate was diluted to 10 ml with diluent.

Screening of Detection Wavelength

The solution was scanned between 190–400 nm using a DAD detector, and 220 nm was chosen as the analytical wavelength because adequate intensity was observed for both peaks.

METHOD VALIDATION

Specificity

Injected blank, placebo, standard, and sample spiked solution into the system and recorded chromatogram for any peaks co-eluting or overlapping with the analyte. The placebo was prepared using the common excipients present in the marketed PIOZ-V tablet formulation, including microcrystalline cellulose, lactose monohydrate, croscarmellose sodium, magnesium stearate, povidone, and colloidal silicon dioxide, without the active pharmaceutical ingredients. The placebo mixture was processed similarly to the sample preparation and filtered through a 0.45 µm membrane filter before injection.

Repeatability & System Suitability

Six replicate injections of the solution are made under the same chromatographic conditions. The system suitability parameters assessed include retention time, theoretical plates (column efficiency), asymmetry factor (tailing factor), and peak resolution.

LOD/ LOQ

The equations calculated the limit of Detection and quantification:

$$\boldsymbol{LOD = \frac{3.3\sigma}{Slope}};\ \boldsymbol{LOD = \frac{10\sigma}{Slope}}$$

Linearity & Range

Solutions of different concentrations, ranging from 40-60 µg/ml for Vildagliptin and 12-18 µg/ml for pioglitazone, were prepared as test concentrations. Peak area was assessed, and a calibration curve was plotted.

Accuracy

Samples were prepared at 80%, 100%, and 120% concentrations by adding 0.8, 1.0, and 1.2 mL of vildagliptin stock solution and 1.2, 1.5, and 1.8 mL of pioglitazone stock solution to separate 10 mL volumetric flasks, then diluting to the mark with diluent. The samples were run in triplicate, and the percentage relative standard deviation (% RSD) was calculated. The amount recovered and the percentage recovery were determined using the following formula.

$$\boldsymbol{Amount\ Recovered = \frac{Au}{As} x\ Spiked\ Conc.\ of\ 100\%}$$

Where,

Au = Peak area of analyte at respective level, As = Peak area of Standard solution,

Robustness

The robustness of the developed method was examined by intentionally varying the column temperature and detection wavelength by ±2°C and ±2 nm, respectively. Under these modified conditions, injections of both the standard and sample solutions were performed, and the percentage relative standard deviation (%RSD) of the assay outcomes was determined for

each variation. This evaluation confirmed the method's stability and reproducibility against minor, deliberate fluctuations in key chromatographic parameters.

Intra & Inter-day Precision

Intra-day precision was determined by preparing the standard and sample solutions and analyzing them twice within the same day at different time intervals. Inter-day precision was evaluated by repeating the analysis on the following day. The percentage relative standard deviation (%RSD) of the assay results was calculated at each interval to assess solution stability over time.

Method Precision

Method precision was evaluated by preparing and injecting six replicate solutions of the drug product sample. The percentage relative standard deviation (%RSD) for both peak area and retention time was calculated and kept within the acceptable limit of 2.0.

Intermediate Precision

Six replicate solutions of the drug product sample were independently prepared and analyzed by different analysts on separate days using the HPLC system. The % relative standard deviation (%RSD) for both peak area & RT was calculated and found to be within the acceptable limit of 2.0.

FORCED DEGRADATION STUDIES

Forced degradation was carried out by preparing the following solutions

Preparation of Standard

5 mg of vildagliptin and 7.5 mg of pioglitazone were accurately weighed and transferred into separate 10 ml and 50 ml volumetric flasks. Diluent was added and mixed thoroughly, and the volume was made up to the mark with diluent to obtain 500 µg/ml vildagliptin and 150 µg/ml pioglitazone.

Control WS

An accurately measured 1 ml portion of the vildagliptin and pioglitazone solution was transferred into a 10 ml volumetric flask, diluted to volume with the diluent, and thoroughly mixed to ensure uniformity.

Acid Degradation

1 mL of each drug solution was transferred into a 10 mL volumetric flask, followed by the addition of 2 mL of 1 M hydrochloric acid. The mixture was maintained at room temperature for 60 minutes and then diluted to volume with the diluent.

Base Degradation

1 mL of each drug solution was placed in a 10 mL standard flask, followed by the addition of 2 mL of 1 M sodium hydroxide. The mixture was allowed to stand at room temperature for 60 minutes and then diluted to the mark with the diluent.

Peroxide Degradation

1 mL of the standard drug solution was transferred into a 10 mL standard flask, followed by the addition of 2 mL of 3% hydrogen peroxide solution. The mixture was kept at room temperature for 60 minutes and then diluted to the mark with the diluent.

Dry heat degradation

Accurately weighed quantities of 5 mg of vildagliptin and 7.5 mg of pioglitazone were transferred into 10 mL and 50 mL volumetric flasks, respectively. The flasks were placed in a hot air oven at 50°C for 4 hours, and then allowed to cool to room temperature. The residues were dissolved in the diluent, and the volume was adjusted to the mark. Subsequently, 1 mL from each stock solution was pipetted into a 10 mL volumetric flask, the flask was diluted to volume with diluent, and the contents were mixed thoroughly.

UV Degradation

To a 10 ml volumetric flask, 5 mg of vildagliptin was accurately weighed and transferred, and to a 50 ml volumetric flask, 7.5 mg of pioglitazone was accurately weighed and transferred. The volumetric flasks were kept in a UV cabinet at short UV (i.e., 254 nm) for 4 hours, then dissolved in diluent and made up to the mark. An additional 1ml of each stock was transferred to a 10 ml volumetric flask & made up to the mark, and mixed well.

RESULT AND DISCUSSION

Experimental Design optimization by CCD

Thirteen experimental trials with varied buffer pH and buffer concentration were carried out using Central Composite Design (CCD). The most important factor affecting resolution was buffer pH; concentration also contributed substantially. The quadratic model's fit to RT, resolution, and theoretical plates' data was highly significant, with an F-value of 3055.23 and a p-value < 0.0001, indicating its remarkable predictive capacity (Table 4). A, B, AB, A^2, and B^2 are significant model terms for the retention time and resolution. The quadratic term A^2 makes

the greatest contribution to the model (Sum of Squares=917.34, F=737.62, p < 0.0001), showing a significant nonlinear (curved) influence of buffer pH on resolution. The interaction term AB is also significant (p=0.0182), indicating that buffer pH and concentration impact resolution. The residual variance is modest (mean square = 1.24), and the lack-of-fit test is non-significant (p = 0.0595), indicating that the model fits the data satisfactorily and without systematic error. The quadratic model for "Theoretical Plates" is statistically significant (F-value = 48.53, p-value < 0.0001), indicating excellent predictive power. Buffer concentration (B) and its interaction with buffer pH (AB) are very significant (p < 0.0001), indicating a strong impact on theoretical plates. The quadratic term of buffer pH (A^2) is significant (p = 0.0045), suggesting a nonlinear connection with theoretical plates. However, the linear term in buffer pH (A) and the quadratic term in buffer concentration (B^2) are not significant (p > 0.05), indicating negligible individual effects.

Table 4: ANOVA for Quadratic model- retention time, Resolution, and Theoretical plates for PIO and VIL

	Retention time		Resolution		Theoretical Plates		
Source	**F-value**	**p-value**	**F-value**	**p-value**	**F-Value**	**P-Value**	
Model	3055.23	< 0.0001	309.20	< 0.0001	48.53	< 0.0001	significant
A-BUFFER PH	12393.71	< 0.0001	26.47	0.0013	0.0035	0.9545	
B-BUFFER CONC	225.60	< 0.0001	224.66	< 0.0001	152.72	< 0.0001	
AB	64.62	< 0.0001	9.39	0.0182	128.53	< 0.0001	
A^2	813.29	< 0.0001	737.62	< 0.0001	16.99	0.0045	
B^2	49.76	0.0002	6.55	0.0376	0.1524	0.7079	
Residual							
Lack of Fit	3.31	0.1765	1.99	8.00	0.7869	0.6027	not significant

Fit Statistics for the model.

The difference between the predicted R^2 and adjusted R^2 values is less than 0.2, indicating satisfactory agreement between them. The signal-to-noise ratio evaluates adequate precision, and a ratio exceeding 4 is considered optimal. This model can therefore be used with confidence to explore the design space shown in Table 5.

Table 5: Fit statistics for the quadratic model

	Retention time	Resolution	Theoretical plates
R^2	0.9995	0.9955	0.9720
Adj. R^2	0.9992	0.9923	0.9519
Pred. R^2	0.9983	0.9702	0.9006
Adeq Precision	140.1563	50.9102	28.1383

Graphical optimization

The contour (Figure 3a) and 3D surface plots (Figure 4a) for retention time show that the buffer pH has the greatest impact on retention time, increasing from pH 3 to 7. There is a modest effect on buffer concentration, most noticeable at higher pH values, where a larger buffer concentration marginally shortens retention time. The curvature of contour lines indicates that pH and buffer concentration interact to affect retention behavior. While the contour plot (Figure 3b) and 3D surface plots (Figure 4b) clearly show that pH and buffer concentration affect resolution, the results indicate a range of ideal resolution values near buffer pH 7 and buffer concentrations of about 60. Both lower and higher pH levels reduce resolution, indicating that pH has a quadratic effect on separation efficiency. Higher buffer concentrations favor better resolution, most likely due to stronger ionic bonds that stabilize analyte interactions. These results validate the proposed method's choice of buffer pH around 7 and concentration of approximately 60 for maximal chromatographic resolution.

According to the contour plot (Figure 3c) and the 3D surface plots (Figure 4c), theoretical plates representing column efficiency are at their highest when the buffer pH is lower (~3) and the buffer concentration is lower (~60 mM), with values exceeding 5000. The shift from warm (yellow/red) to cool (blue) hues indicates a reduction in column efficiency as pH and buffer concentration increase. These findings imply that, under the settings studied, higher chromatographic efficiency is favored by an acidic pH and a lower ionic strength, but resolution decreases when the pH falls to 3. This information makes it easier to choose operational conditions logically to maximize column performance. The graph overlay (Figure 5) validates the graphical optimization and creates a single figure showing the location where the response requirements can be satisfied. The acceptable factor parameters are defined as yellow by default. The undesirable factor settings are indicated in a different color, which is grey by default. A combination of acceptable and unacceptable colors is used to indicate where the interval limitations are inappropriate if intervals are part of the criterion.

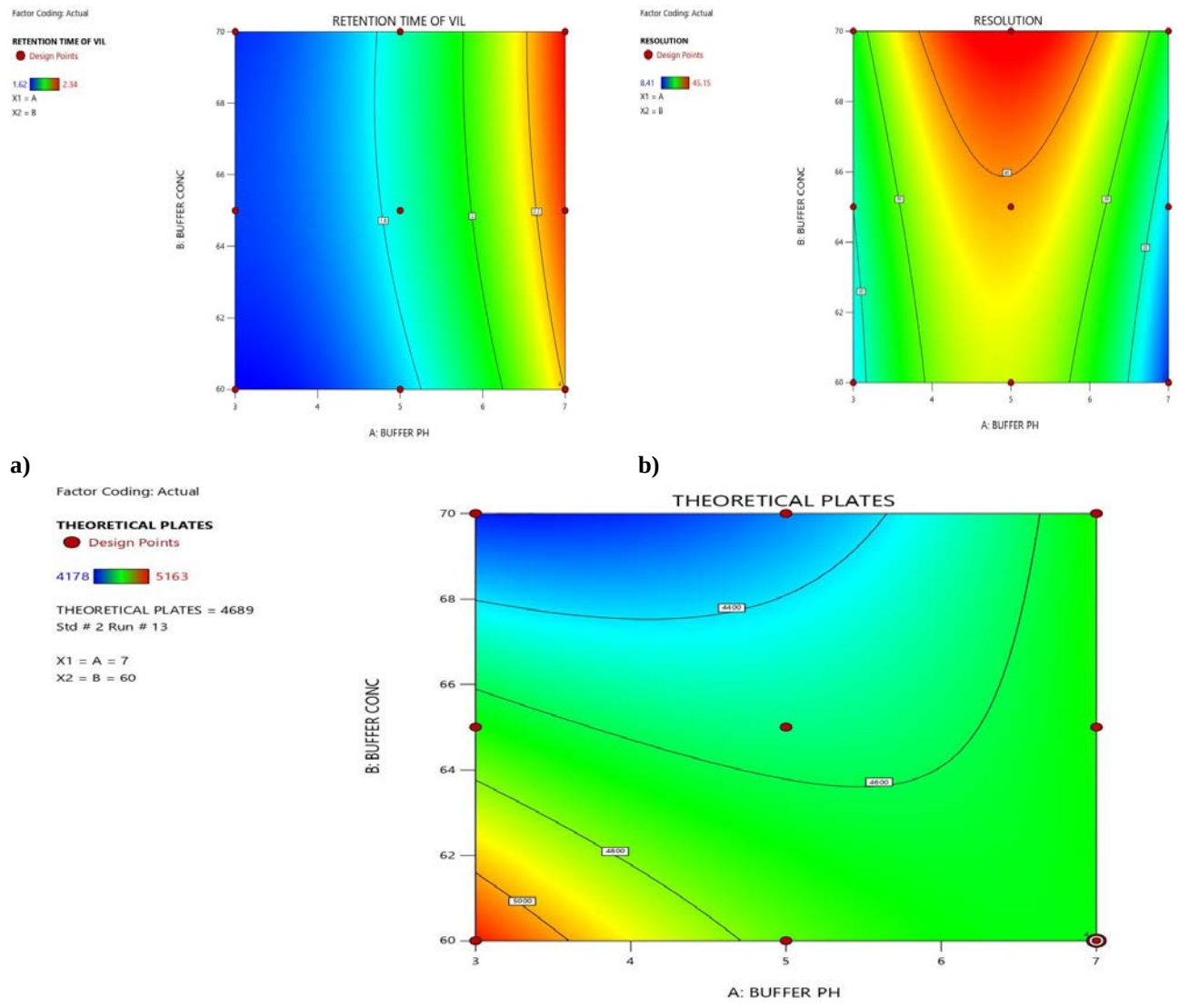
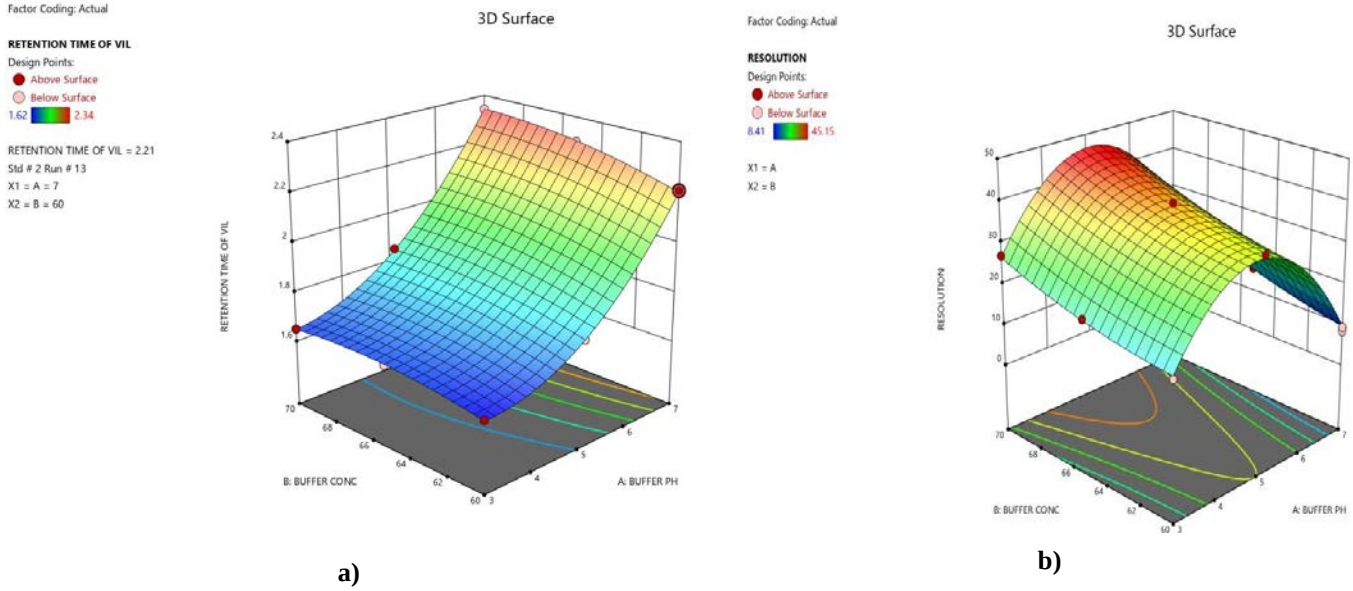
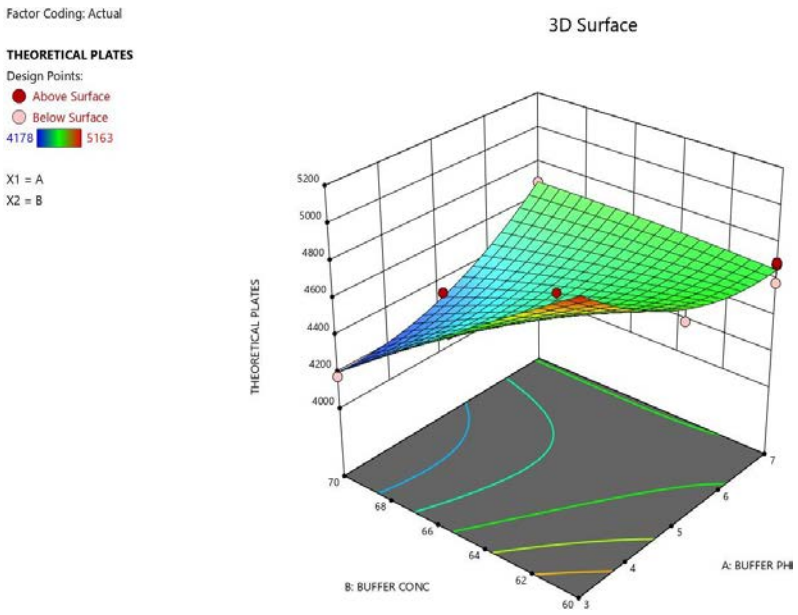


Figure 3: a, b, c Contour plots showing the interaction of CMP with CQA





c)

Figure 4: a, b, c 3D Surface methodology plot showing interaction of variables

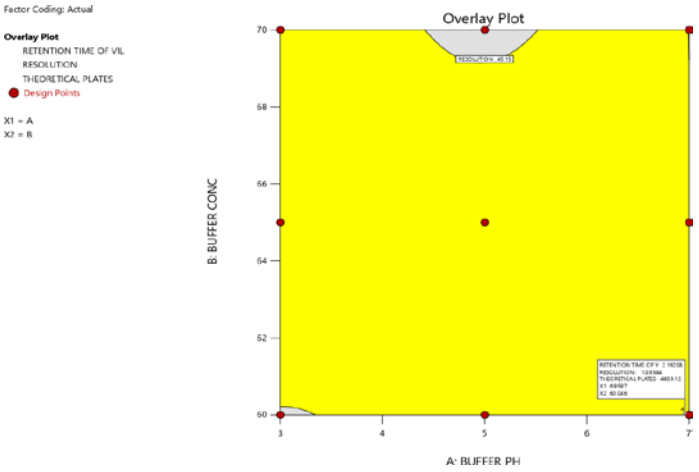


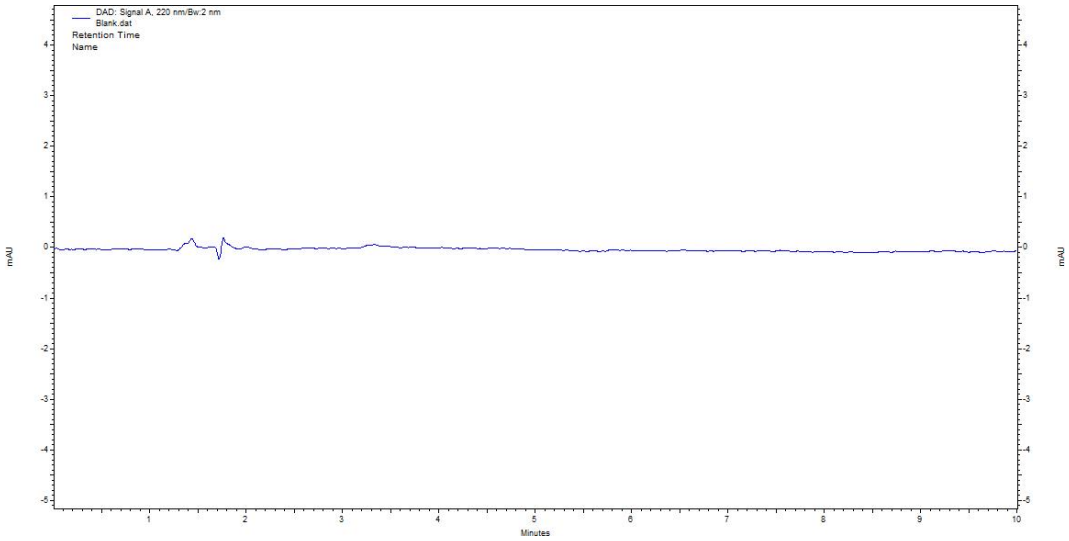
Figure 5: Overlay plot showing Acceptable factor settings

Method validation

Specificity

No interference peaks were obtained until the analyte's retention time. The chromatograms showed that neither the blank solution nor the excipient solution had any peaks during the analyte's retention time. The method's selectivity was further demonstrated by forced degradation investigations, which showed that all notable degradation products were effectively isolated from the analyte.

These findings demonstrate the specificity of the devised RP-HPLC technique for quantifying the analyte in the presence of common sample components.



a)

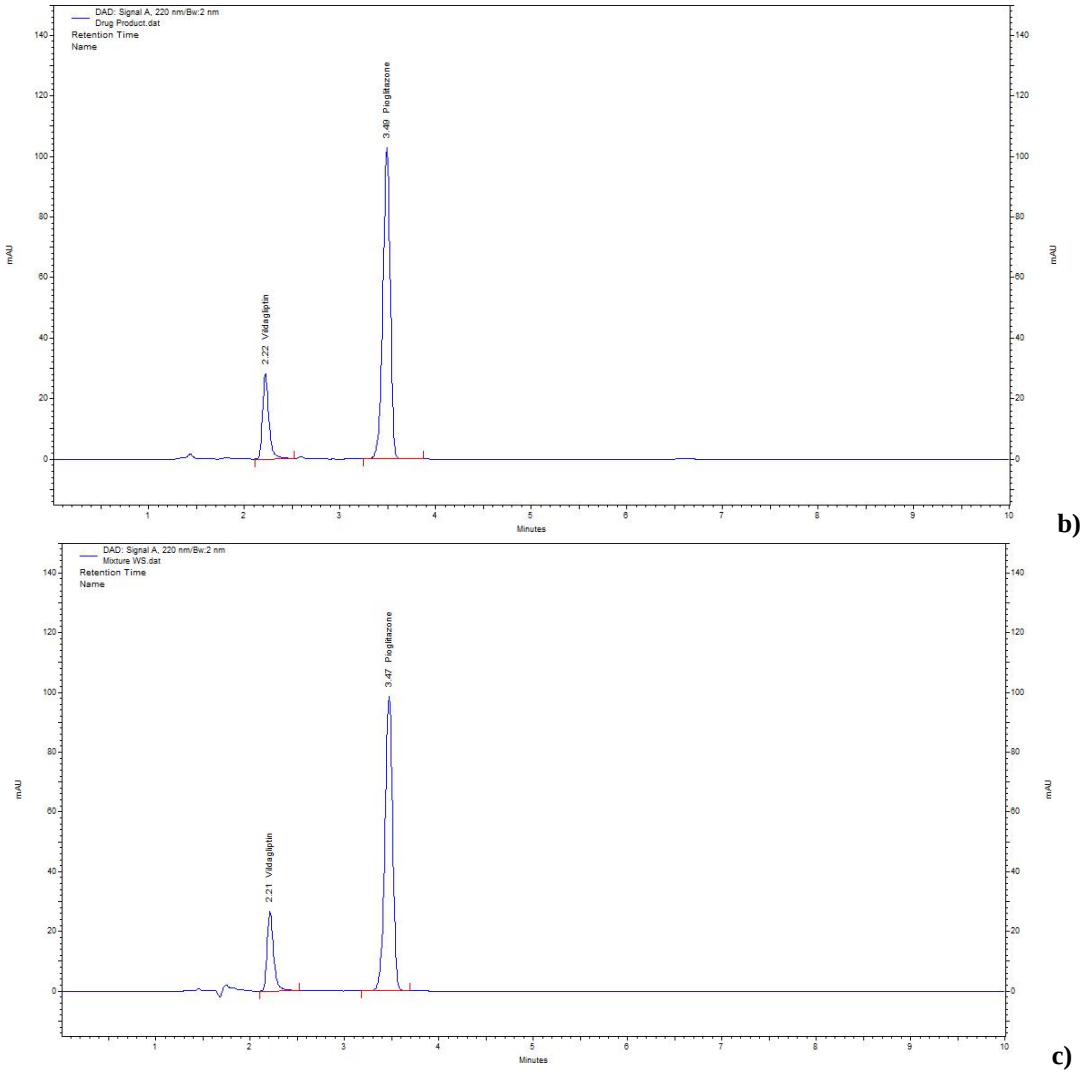


Figure 6: Chromatogram of a) Blank solution, b) PIO and VIL standard mixture, and c) Marketed formulation at optimized chromatographic conditions

Repeatability and system suitability

Repeatability and system suitability were assessed by injecting the working standard solution 6 times and evaluating the retention time (RT), theoretical plates (TP), resolution (RES), and tailing factor or asymmetry (ASY). The theoretical plate count is not less than 2000, and the tailing factor is within 0.5-1.5. The percentage relative standard deviation (RSD) of peak area and retention time is 0.54 and 0.01 for VIL and 0.16 and 0.30 for PIO, respectively, and is less than 2; these values were among the system suitability metrics that met the ICH recommendations. The data is shown in Table 6.

The symmetrical peak shape and effective column performance are shown by the low tailing factor & high theoretical plate number. Fulfilling these requirements before sample analysis confirms that the chromatographic instrument is operating reliably and that the technique can separate & quantify data effectively. Reliable method operation & continuous instrument qualification are supported by consistent system suitability performance throughout validation runs.

Linearity and Range

Linearity was observed within the concentration ranges of 40-60 µg/ml for Vildagliptin and 12-18 µg/ml for pioglitazone. The R^2 value is 0.99, within the acceptable range, and the regression equations are y = 5452.1x – 3870 for vildagliptin and y = 78736x + 1408.8 for pioglitazone. The overlay linearity plot for the chromatogram is shown in Figure 9.

LOD AND LOQ

The limit of detection and quantification was calculated using the equation, which yielded 1.93 and 5.85 µg/ml for Vildagliptin and 0.32 and 0.97 µg/ml for pioglitazone.

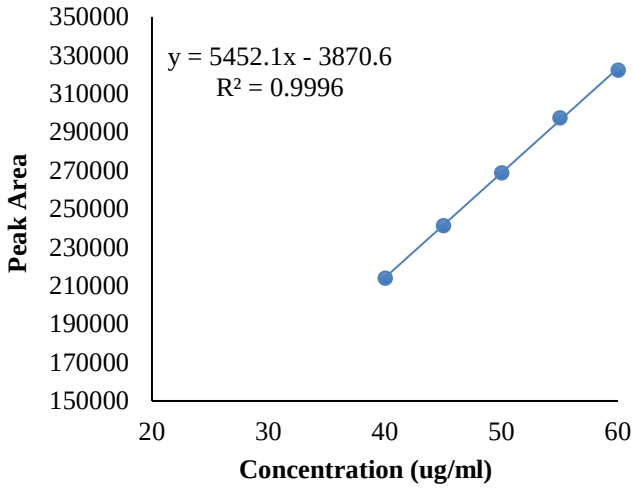


Figure 7: Linearity curve of Vildagliptin

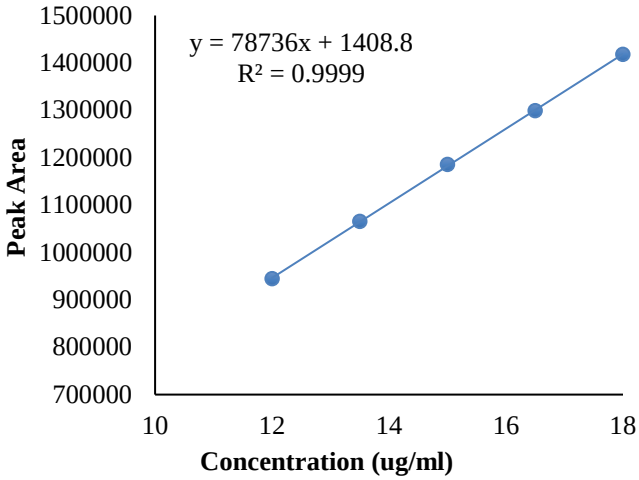


Figure 8: Linearity curve of Pioglitazone

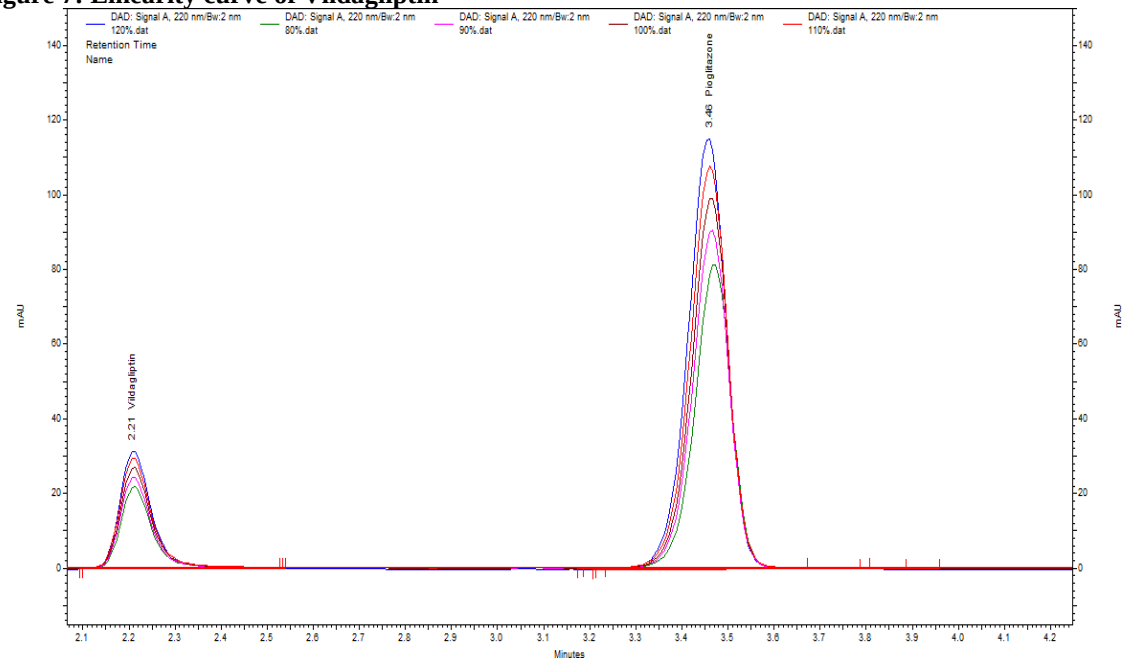


Figure 9: Chromatogram showing linearity

Accuracy

Recovery trials employing the standard addition procedure at three concentration levels (80%, 100%, and 120% of the label claim) were used to assess the accuracy of the proposed RP-HPLC method. Every level was examined three times. The mean percent recoveries for all tested concentrations ranged from 98.83% to 100.12% for VIL and 99.26% to 100.02% for PIO, with %RSD <2%. These data show that the approach is quite accurate for quantification in pharmaceutical formulations, since they fall within the ICH-accepted range of 95–105%. The method's suitability for routine analysis was confirmed by results consistent with those of earlier studies on comparable analytes. Since recoveries at all levels were comparable, no discernible matrix impact was found.

Intermediate Precision

The intermediate precision was assessed by evaluating samples within the days and on different days under the same chromatographic conditions. The assay findings for the formulation were consistent and repeatable across all settings evaluated. The percentage relative standard deviation (%RSD) for intermediate-precision investigations was less than 2%, indicating high precision between days. The test results remain near the target concentration, indicating the method's reliability and robustness.

Method Precision

Six injections of the drug product were prepared and injected, and the % RSD for peak area, retention time, theoretical plates,

asymmetry, and resolution were analyzed. These parameters are well within the acceptable limits and shown in Table 6.

Robustness

Robustness is determined by deliberately varying factors such as column temperature (±2°C), detection wavelength (±2 nm), minor variations in flow rate (±0.1 mL/min), and the organic phase composition of the mobile phase (±2%). The findings showed minor variability in chromatographic performance, with percent RSD values for peak area and retention time within the permitted 2% level, as shown in Table 6. These findings revealed that the approach is robust enough to withstand small but purposeful changes in chromatographic conditions without affecting accuracy, precision, or system appropriateness.

Table 6: RP-HPLC method-overall validation report

Parameters		Vildagliptin	Pioglitazone	Acceptance
RT(min)		2.21min	3.47 min	-
LOD (µg/ml)		1.93 µg/ml	0.32 µg/ml	-
LOQ(µg/ml)		5.85µg/ml	0.96 µg/ml	
Linearity(µg/ml)		40-60 µg/ml	12-18 µg/ml	-
Accuracy(% Recovery)		98.83 -100.12%	99.53 -99.98 %	90-110%
System suitability (%RSD)		0.37	0.30	<2%
Repeatability (%RSD)		0.54	0.16	<2%
Precision (% RSD)	Method	0.34	0.23	<2%
	System	1.15	0.33	<2%
	Intra day	0.52	0.66	<2%
	Inter day	1.22	0.59	<2%
Robustness				
Wavelength (%RSD)		0.34	0.22	>2%
Column Temperature (%RSD)		0.23	0.23	>2%
Flow rate % (RSD)		0.50	1.13	>2%
Organic phase composition(% RSD)		0.56	1.56	>2%
Specificity	No peaks detected up to run time of 10 min			No peaks

Forced Degradation studies

The degradation profiles for both drugs showed slight degradation relative to the control. The control demonstrated a 100% pass rate, serving as the baseline and indicating no deterioration under normal circumstances. Pioglitazone degraded minimally under acidic, thermal (dry heat), and UV environments, with percent degradation values well below 2%, demonstrating good stability under these conditions. Pioglitazone was most susceptible to deterioration under basic conditions (6.80%), followed by oxidative (peroxide) stress (4.20%), consistent with prior results indicating that the molecule is more susceptible to alkaline hydrolysis and oxidation. Dry heat and UV conditions exhibited practically minimal degradation, indicating that the compound is thermally and photochemically stable.

The acid-treated vildagliptin sample showed an assay of 102.35%, slightly above the control. This negative degradation score (-2.35%) indicates no actual loss and may be due to experimental variability, interference, or an extremely small assay overestimate. It demonstrates that Vildagliptin is extremely stable under acidic environments. The base-stressed sample showed a considerable decrease in the assay to 81.62%, corresponding to 18.38% degradation. This indicates Vildagliptin's severe instability under basic circumstances, most likely owing to hydrolysis or structural decomposition. Under oxidative circumstances, the test yielded 97.60% (2.40% degradation), indicating moderate susceptibility to peroxides. Exposure to dry heat produced 98.31% assay (1.69% degradation). This demonstrates good thermal stability, with just minor losses under heat stress. The UV sample demonstrated 98.69% assay and 1.31% degradation, showing resistance to photolytic degradation and high photostability. Vildagliptin is stable under acidic, thermal, and photolytic stress, with minimal degradation detected. Base stress causes the most significant deterioration (18.38%), indicating that it is sensitive to alkaline conditions. Peroxide causes little degradation, as does heat and UV, indicating a generally robust stability profile unless under extreme alkaline stress. It is shown in Table 10 and Figure 9. These results demonstrate the proposed RP-HPLC method's stability-indicating capabilities, as pioglitazone and vildagliptin were clearly separated and quantified even in the presence of

degradation products. The observed degradation profile matches previous literature, in which alkaline and oxidative stressors are often the most harmful to medication compounds such as pioglitazone and vildagliptin, whereas acid, heat, and light cause very minimal changes. Peak purity analysis was performed using a PDA detector for all stressed samples. UV spectra were extracted at the start, apex, and end of each analyte peak to confirm spectral homogeneity. The purity angle was below the purity threshold under all stress conditions, indicating the absence of co-eluting degradation products, as shown in Figure 11. These findings confirm the stability-indicating capability of the developed RP-HPLC method.

Table 7: Degradation studies of pioglitazone and vildagliptin

Sample ID	Vildagliptin				Pioglitazone			
	Peak Area	% Assay	% Deg	Mass Balance (%)	Peak Area	% Assay	% Deg	Mass Balance(%)
Control WS	268819	100	-	100	1133290	100	-	100
Acid Sample	275144	102.35	-2.35	100	1119059	98.74	1.26	100
Base Sample	219401	81.62	18.38	100	1056236	93.20	6.80	100
Peroxide Sample	262356	97.60	2.40	100	1085670	95.80	4.20	100
Dry Heat Sample	264268	98.31	1.69	100	1132266	99.91	0.09	100
UV Sample	265309	98.69	1.31	100	1125298	99.29	0.71	100

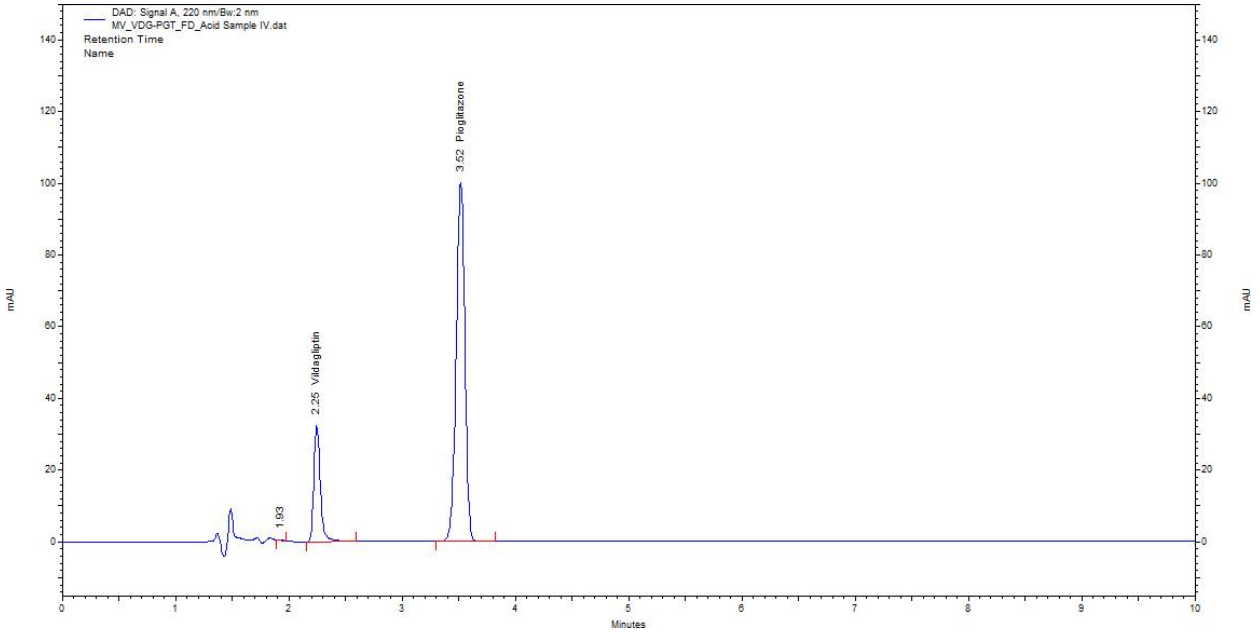
Assessment of the greenness of the analytical method

According to the AGREE, MoGAPI, and BAGI tools, the method greenness evaluation for the RP-HPLC analysis shows a generally positive environmental profile, with particular actionable insights highlighted for future improvement. With a score of 0.66, the AGREE metrics indicate moderate adherence to the 12 Green Analytical Chemistry principles, as shown in Figure 12a.

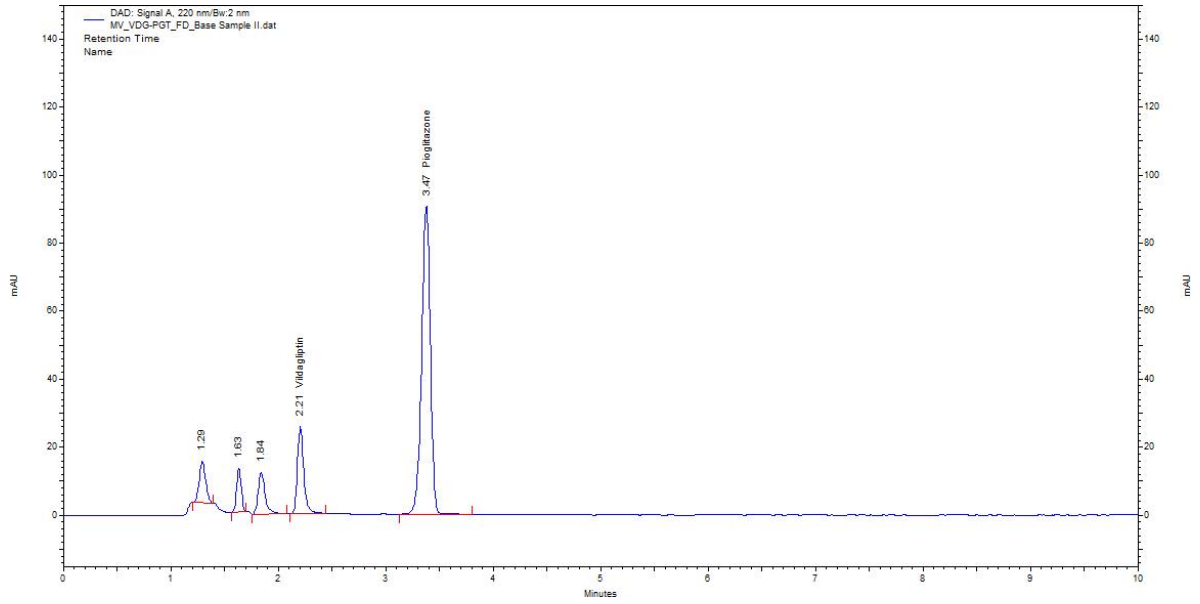
The visual breakdown indicates that the majority of the principles (green/yellow sectors) were satisfactorily followed; nevertheless, the red-highlighted sections (principles 9 and 12 in particular) indicate scope for development, possibly in areas such as waste minimization or energy use. The MoGAPI value is 74, illustrated in Figure 12b, indicating that the technique is greener than average within the modular evaluation criteria. Different modules are colored red or yellow, suggesting specific technique components (such as sample preparation, reagent selection, or equipment) that are less sustainable; an emphasis on eliminating toxic solvents and optimizing process steps might further boost the score.

The Blue Applicability Grade Index (BAGI) has a high score of 82.5(Figure 12c), indicating good overall technique applicability and environmental performance in terms of analytical appropriateness, safety, and sustainability. With the blue shading signifying compliance and preparedness for widespread adoption, including regulatory and scalability issues, the pentagonal map exhibits balanced strengths across all assessed categories.

a.



b.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MV_VDG-PGT_FD_Base Sample II.dat
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Name
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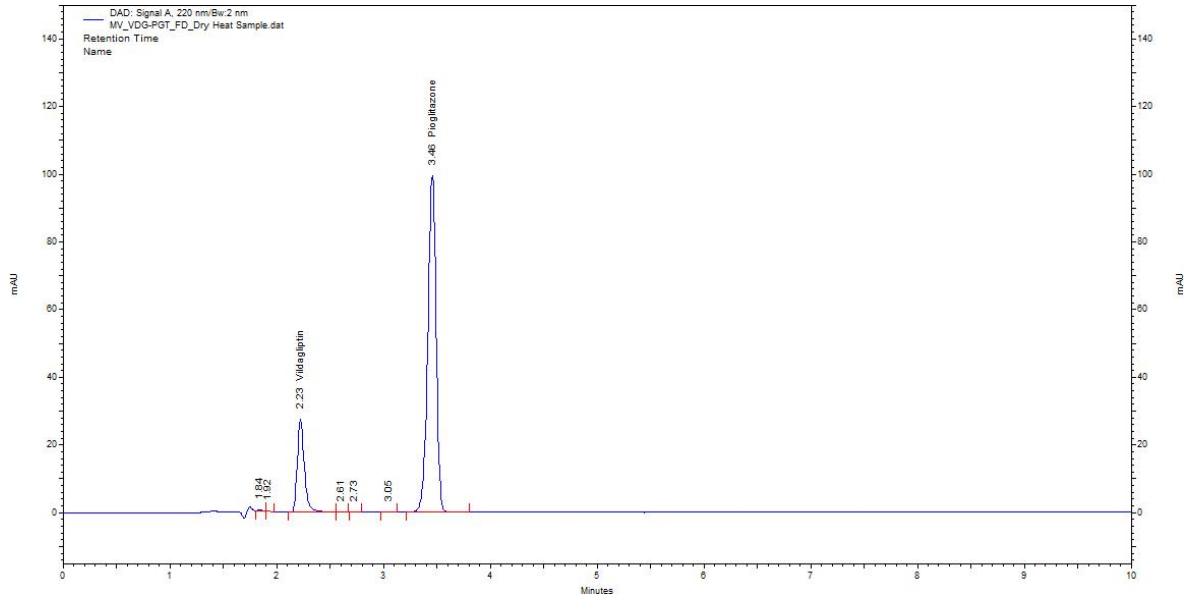
1.63
1.84
2.21 Vildagliptin
3.47 Pioglitazone
mAU
Minutes

c.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Retention Time
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3.46 Pioglitazone
mAU
Minutes

d.

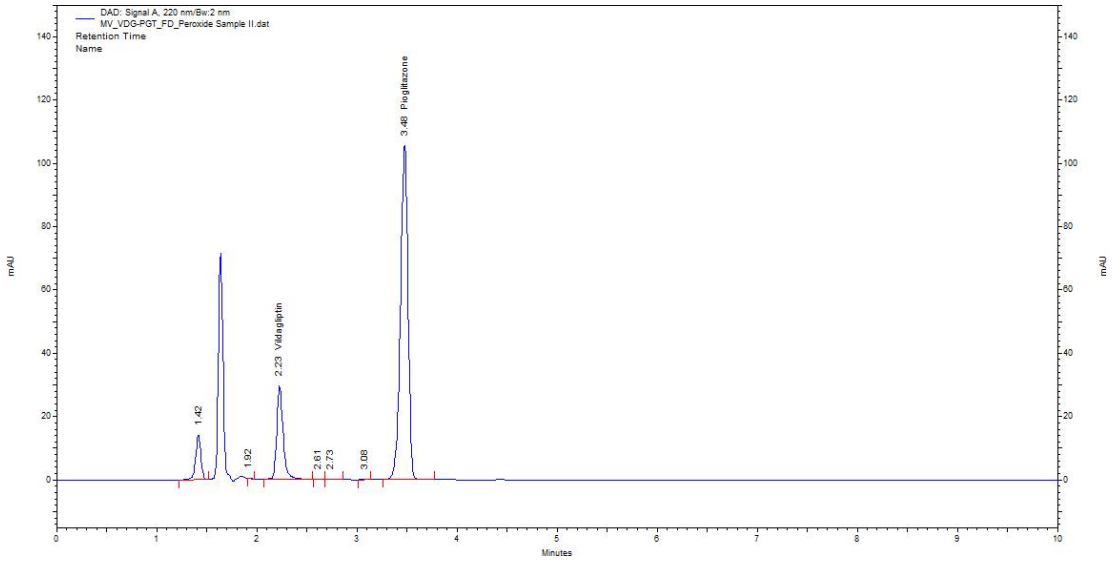

Figure 10: chromatogram of a) acid, b) base, c) dry heat, d) UV, and e) oxidation-degraded sample of PIO AND VIL

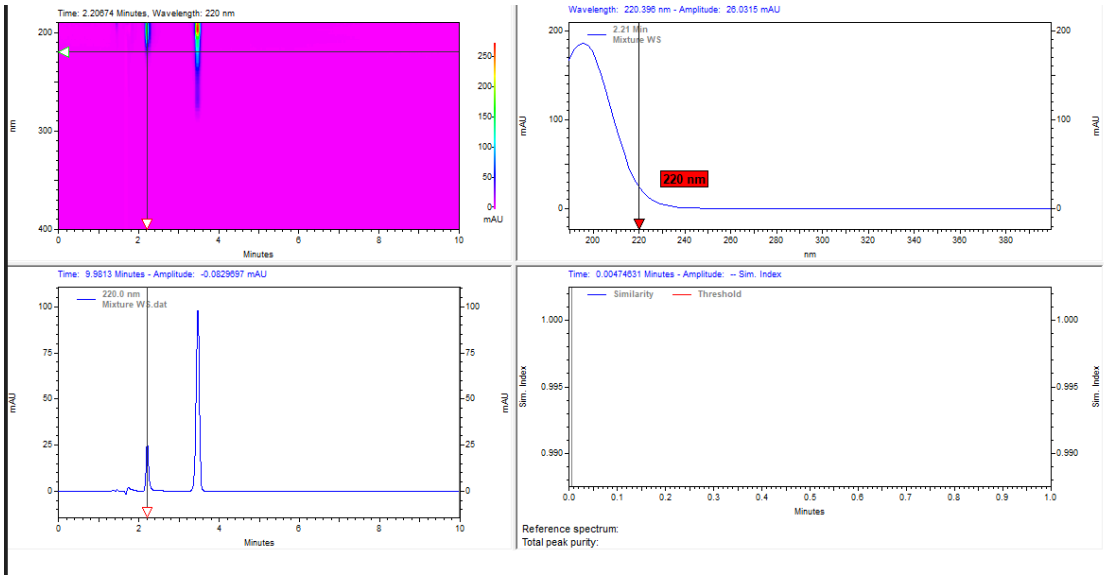

Figure 11: PDA Peak Purity Analysis of Stressed Sample

a) AGREE **b) MoGAPI** **c) BAGI Index**

Figure 12: Green Assessment and BAGI score of the developed method

CONCLUSION

A green, stability-indicating RP-HPLC method based on an Analytical Quality by Design (AQbD) approach was successfully developed for the simultaneous estimation of pioglitazone and vildagliptin. Central Composite Design (CCD) clearly identified buffer pH and buffer concentration as critical factors, with buffer pH exerting the strongest influence on retention time and resolution. The developed quadratic models

demonstrated excellent statistical significance, high predictability, and adequate precision, enabling reliable optimization of the method within a defined design space. The optimized method demonstrated high specificity, accuracy, precision, robustness, and sensitivity in accordance with ICH guidelines. Linearity was achieved over the selected concentration ranges, and low LOD and LOQ values confirmed suitability for routine quality control. The total runtime of the final optimized method was 10 minutes, reducing analysis time and improving analytical throughput and operational efficiency. Forced degradation studies confirmed the method's stability-indicating capability, with both drugs effectively separated from their degradation products. Alkaline and oxidative conditions caused the most degradation, consistent with reported literature.

Greenness evaluation using AGREE, MoGAPI, and BAGI tools indicated a favorable environmental profile with good analytical applicability. Although the method employs conventional solvents with comparatively low acetonitrile concentration and instrumentation, it offers scope for future improvements in solvent reduction. Overall, this study highlights the importance of integrating AQbD with green analytical assessment to develop robust, reliable, and sustainable analytical methods. The proposed RP-HPLC method is well-suited for routine quality control and stability testing of pioglitazone and vildagliptin pharmaceutical formulations in the current regulatory and sustainability-focused analytical environment.

FINANCIAL ASSISTANCE
NIL

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Devi Swapna PV performed the laboratory work and drafted the manuscript, while G. Saravanan supervised the study and reviewed, corrected, and approved the final version of the manuscript**.**

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