Journal of Applied Pharmaceutical Research
Volume 14 Issue 3, Year of Publication 2026, Page 122 – 130
DOI: https://doi.org/10.69857/joapr.v14i3.1914

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Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | JOAPR
www.japtronline.com ***ISSN: 2348 – 0335***

AQBD ASSISTED UPLC METHOD DEVELOPMENT AND VALIDATION OF TIRZEPATIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Article Information
Received: 14th January 2026
Revised: 29th March 2026
Accepted: 1st May 2026
Published: 15th May 2026

Keywords
Tirzepatide, UPLC, Diabetics, AQbD, Validation.

ABSTRACT

Background: Tirzepatide is a USFDA-approved (2023) synthetic drug that primarily targets blood sugar metabolism for chronic weight management, obesity-related conditions, and type 2 diabetes. In this study, we aim to develop a reliable, sensitive UPLC method for estimating Tirzepatide using Analytical Quality by Design (AQbD) to optimize chromatographic conditions. **Methodology:** The finalized method was developed using the Agilent 1290 Infinity II LC System, with a Phenomenex C18 column (50 x 1.7 mm, 2.1 µm), using a mobile phase of acetonitrile and trifluoroacetic acid buffer (37.07:62.93, v/v) at a flow rate of 0.57 mL/min and a Photo Diode Array detector at 264 nm. **Result and Discussion:** The method validation showed linearity over the range of 12.5–75 µg/mL (R^2 = 0.9995). The intraday and interday precision (%RSD) values were 0.603 and 0.791, respectively, confirming the method's reproducibility. Limit of Detection and Limit of Quantification values were calculated from S/N ratios of the prepared samples and were 0.6 and 2, respectively. Accuracy was validated to be 99.3-101.1%. The forced degradation studies were conducted under stress conditions, including acid, alkali, peroxide, reduction, photodegradation, and hydrolysis, with tirzepatide showing degradation percentages of 1.3%, 11.3%, 12.1%, 2.1%, 1.7%, and 2.9%. **Conclusion:** The developed UPLC method for quantifying tirzepatide was found to be significant, with all evaluated parameters in agreement with ICH guidelines. The proposed method is efficient, straightforward, and dependable, rendering it appropriate for routine quality control of tirzepatide in bulk and pharmaceutical formulations.

INTRODUCTION

The 21st century is seeing a sharp rise in the prevalence of diabetes mellitus (DM) due to several factors, including obesity, aging populations, inactivity, and a rise in the migration of vulnerable patients. Recently, this persistent and expensive illness has been compared to the 14th-century "Black Death." The main goal of managing type 2 diabetes, which is more prevalent, is to achieve adequate glycaemic control to postpone microvascular and macrovascular consequences. This procedure includes medication therapy as well as lifestyle modifications, including exercising and losing weight. A greater understanding of the biology of diabetes has led to the development of new

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medications, such as insulin analogs, thiazolidinediones (TZDs), dipeptidyl peptidase-4 (DPP-4) inhibitors & glucagon-like peptide-1 (GLP-1) mimetics. While DPP-4 inhibitors prevent the endogenously produced hormone from being inactivated, GLP-1 agonists replicate the effects of this incretin. Although additional research is required to validate their safety and clinical role, both agents offer an effective alternative to the hypoglycaemic medications currently on the market [1]. Tirzepatide (Figure 1), an anti-diabetic drug used to treat type 2 diabetes, exerts its therapeutic action by binding with the gastric inhibitory-ry polypeptide (GIP) receptor and glucagon-like polypeptide-I (GLP-I). GIP receptors play an important role in blood glucose regulation; they are present on the surface of pancreatic beta cells and, upon binding glucagon-like peptide-1, stimulate insulin secretion. GLP-1 helps increase insulin secretion from the pancreas. Tirzepatide targets both the GIP and GLP-1 receptors, thereby regulating blood sugar levels. Chemically, its structure consists of 39 amino acids and a 20-carbon fatty diacid residue; this polypeptide sequence is similar to endogenous GIP produced in our body [2], [3].

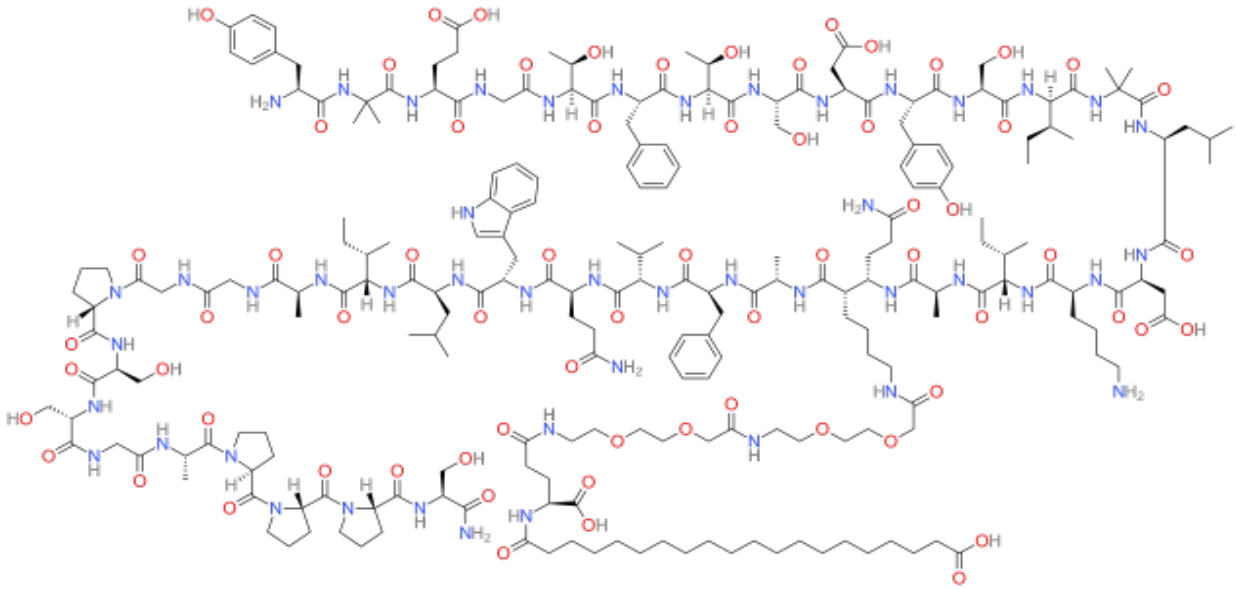

Figure 1: Structure of Tirzepatide

Analytical quality design has recently become a crucial element of the pharmaceutical product development cycle, as it generates reliable, cost-effective, sensitive, robust, and precise analytical methods that can be utilized at all stages of the process [4]. These crucial elements establish a methodical approach. The focus on Analytical Quality by Design (AQbD) involves pinpointing the Analytical Target Profile (ATP) and Critical Method Variables (CMVs) via risk assessment.

The application of Analytical QbD significantly enhances efficiency by minimizing variability and ensuring high robustness and performance throughout the product life cycle, including method development, validation, and transfer [5], [6]. A UPLC approach for tirzepatide has been documented by Chandana Manepalli et al., where they developed a UPLC method using a mobile phase (methanol: acetonitrile: 0.1% sodium dihydrogen orthophosphate in 70:10:20). Separation was performed on an Acquity UPLC BEH C18 (50mm × 2.1 mm, 1.7 µm), with a runtime of 3 min. Our study aims to develop a sustainable, adaptable, and efficient method for routine analysis; specifically, we intend to create a fast, simple, precise, accurate, and sensitive UPLC method by integrating the AQbD approach.

MATERIALS AND METHODS

Chemicals

To perform UPLC analysis, Tirzepatide reference standards (99% purity) were obtained from Biocon Ltd., Bengaluru, India. All solvents used in the analysis were analytical grade. 0.1% Trifluoroacetic acid (Merck), acetonitrile (Merck), and water of HPLC quality (Milli Q or similar).

Instrumentation, apparatus, and software

The Agilent 1290 Infinity II LC System, equipped with a Phenomenex C18 column (50 x 1.7 mm, 2.1 µm), an Alliance

low-pressure gradient pump, a degassing unit, an autosampler, an injector, and a photodiode array detector (2998), facilitated liquid chromatographic separation. Data collection and processing were conducted using Empower 2.0 software. Samples were filtered through 0.22 µm membrane filters, sonicated (Unichrome), and measured with a pH meter (Eutech). For Analytical Quality by Design (AQbD) development, the trial version of Design-Expert® 12 was utilized.

Chromatographic conditions

After initial trials, the final chromatographic separation was achieved on Phenomenex C18 (50 x 1.7 mm, 2.1 µm) at ambient temperature and a mobile phase composed of acetonitrile and 0.1% trifluoroacetic acid (37.07:62.93 v/v) at a flow rate of 0.57 mL/min, and the photodiode array detector was set at 264 nm based on the maximum absorbance of tirzepatide.

Preparation of standard solutions

Prepare a tirzepatide standard solution. 10 milligrams of tirzepatide was dissolved in 10 milliliters of diluent (acetonitrile and 0.1% Trifluoroacetic acid in a 30:70 ratio), yielding a 1000 µg/ml stock solution. Serial dilutions ranging from 12.5 µg/ml to 75 µg/ml were prepared for further analysis [7, 8].

Tirzepatide vials (3 mg) were added to a 100 ml volumetric flask, followed by the addition of diluent (acetonitrile and 0.1% Trifluoroacetic acid in a 30:70 ratio). Then, the solution was sonicated for 30 minutes, filtered, and the filtrate was examined. The assay was carried out twice, and the outcomes closely match the label's assertion.

Experimental design

A risk assessment was conducted to analyze the factors that significantly affect the UPLC method's output. Initially, three potential variables were identified during the preliminary risk assessment: mobile-phase composition (ACN ratio), flow rate, and column temperature. However, screening studies demonstrated that column temperature had minimal influence on critical analytical attributes (CAAs) such as retention time, theoretical plates, and tailing factor within the studied range.

Therefore, it was fixed at an optimized constant level, and only the ACN ratio and flow rate were selected as Critical Method Variables (CMVs) for optimization using CCD. For the analysis and optimization, a three-factor fractional factorial central composite design (CCD) was used as an appropriate experimental design for the variables in question.

Method Validation

In accordance with ICH Q2(R1) guidelines, the new technique was validated under optimal conditions. Linearity was established by analyzing samples across different concentration ranges. The linear curve within the analyzed concentration range was plotted as concentration vs area response. Precision was assessed by repeating measurements of six replicate samples at 50 µg/mL tirzepatide and evaluated as intraday and interday precision [9]. The intraday precision was assessed by analyzing the solution six times with 2h intervals on the same day. In contrast, the same-level solution was assessed six times over two days to assess interday precision. The relative standard deviation (%RSD) of the area response was determined, and values less than 2% were considered acceptable. Accuracy was determined through recovery studies performed over a concentration range of 80, 100, and 120 µg/mL within the linearity range. The % recovery within the 98–102% ICH guideline range was considered accurate. The LOD and LOQ represent the method's sensitivity for detection and quantification of tirzepatide [10], which was calculated by utilizing the standard deviation of the y-intercept (σ) and slope (SD) from the calibration curve by substituting the formulas LOD = 3.3 × σ/SD and LOD = 10 × σ/SD. The system suitability was confirmed by analyzing parameters such as theoretical plates and tailing factor across six replicate analyses of tirzepatide at a precision-level concentration.

Forced degradation studies

Concentrated hydrochloric acid (1N HCl), sodium hydroxide (1N NaOH), hydrogen peroxide (10% H_2O_2), sodium bisulphate (10% $NaHSO_4$), and deionized water were employed as reagents for acid, alkali, peroxide, reduction, and hydrolysis, respectively, to conduct forced degradation studies under stress conditions in compliance with ICH guidelines Q1A (R2). Following the formation of a mixed standard, it was treated for 20 minutes at 60 °C with 1N HCl, 1N NaOH, 10% H_2O_2, and 10% $NaHSO_4$, in that order. 0.5 mg of tirzepatide was exposed to illumination in a photolytic chamber for 3 hours to facilitate photolysis. 0.5 mg of tirzepatide was heated at 105°C for 3 hours to induce thermal degradation. Each sample was ultimately diluted with a diluent to achieve the requisite concentration before analysis by the validated UPLC method [11, 12, 13].

RESULTS AND DISCUSSION

The wavelength for tirzepatide detection was selected by scanning 200-400 nm with a photodiode array detector, and a

peak at 264 nm was observed. In the initial trials, Waters Acquity C18 (100x2.1mm, 1.6 µ) was used as the stationary phase, with acetonitrile and formic acid (30:70) and (40:60) as mobile phases, at a flow rate of 0.5 ml/min. These conditions resulted in more retention time and a broad peak. Then the mobile phase composition was changed from formic acid to trifluoroacetic acid, keeping acetonitrile constant in a 50:50 ratio, resulting in an insufficient baseline. Then acetonitrile and trifluoroacetic acid in 40:60 and 50:50 ratios were used on the Phenomenex C18 stationary phase (50 x 1.7 mm, 2.1 µm), resulting in asymmetric peaks (Figure 2). So, further method optimization was performed using the central composite design.

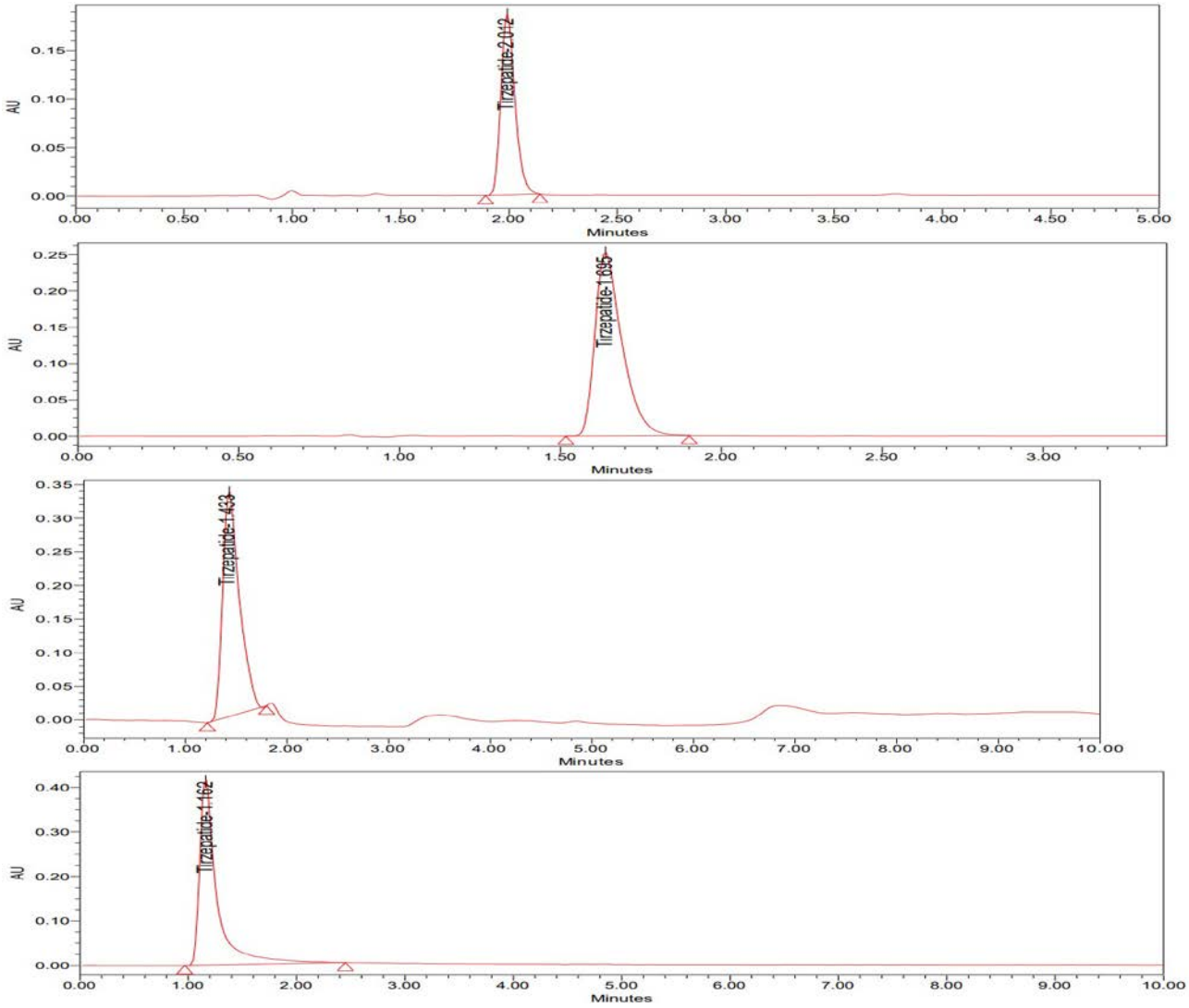

Figure 2: Chromatograms obtained during optimization of Tirzepatide

Central Composite Design (CCD)

CCD in AQbD helps analyze the effects of factors (ACN and flow rate) on the responses (RT, TP, TF) with fewer trial runs than other designs. The software [10] suggested 13 experimental runs**,** as shown in Table 1.

Analysis of the variables

DoE expert® trial version 12 software was used to analyze the data, and an appropriate mathematical model was used. The selected model was found to be significant, with both the adjusted and predicted r^2 values in good agreement. In QbD, the correlations between the factors and each response were analyzed using perturbation plots, 2D contours, and 3D response surface plots. The perturbation plot for retention time (RT) shows that as the ACN ratio increases, RT gradually decreases, whereas flow rate shows no significant change. The perturbation plot for plate count shows that as the ACN ratio increases, TP increases drastically; in contrast, flow rate showed no significant change. The perturbation plot for the tailing factor (TF) shows that as the ACN ratio increases, the TF decreases sharply; in

contrast, the flow rate showed no significant change (Figure 3a). All the 2D contour plots (Figure 3b) for the responses show the same. DoE expert® trial version 12 software was used to analyze the data, and an appropriate mathematical model was used. The selected model was found to be significant, with both the adjusted and predicted r^2 values in good agreement.

Table 1: CCD experiment design for UPLC,

Run	Factor 1	Factor 2	Response 1	Response 2	Response 3
	Acetonitrile	Flow rate	Retention time	Theoretical plates	Tailing factor
1	30.00	0.40	1.17	1.573	4533
2	37.07	0.43	0.96	1.069	7365
3	22.93	0.57	1.10	1.358	5812
4	30.00	0.50	1.02	1.285	6729
5	37.07	0.57	0.97	0.922	8121
6	30.00	0.60	0.91	0.963	8013
7	20.00	0.50	1.22	1.483	3962
8	40.00	0.50	0.90	0.857	9663
9	30.00	0.50	1.08	1.286	6745
10	30.00	0.50	1.01	1.282	6781
11	30.00	0.50	1.06	1.285	6723
12	30.00	0.50	1.03	1.281	6750
13	22.93	0.43	1.28	1.743	2865

Optimization of the UPLC method

For any method development using UPLC, the main objective would be faster analysis with lower RT, higher TP, and lower TF. Hence, the goals were set, and the method was optimized in the software. The ACN ratio and flow rate were kept within range, with RT and TF minimized and TP maximized. The optimized 3D response plot (Figure 3c), analyzed using Derringer's desirability function, indicates lower TF.

Method validation for chromatographic analysis

The system's effectiveness was assessed by measuring characteristics, including theoretical plates (N), tailing, and elution time, using a standard solution of Tirzepatide at 100 µg/ml, which was injected six times. The findings presented in Table 2 fell within the established parameters, with theoretical plates (N) exceeding 2000 and a tailing factor of less than 2.

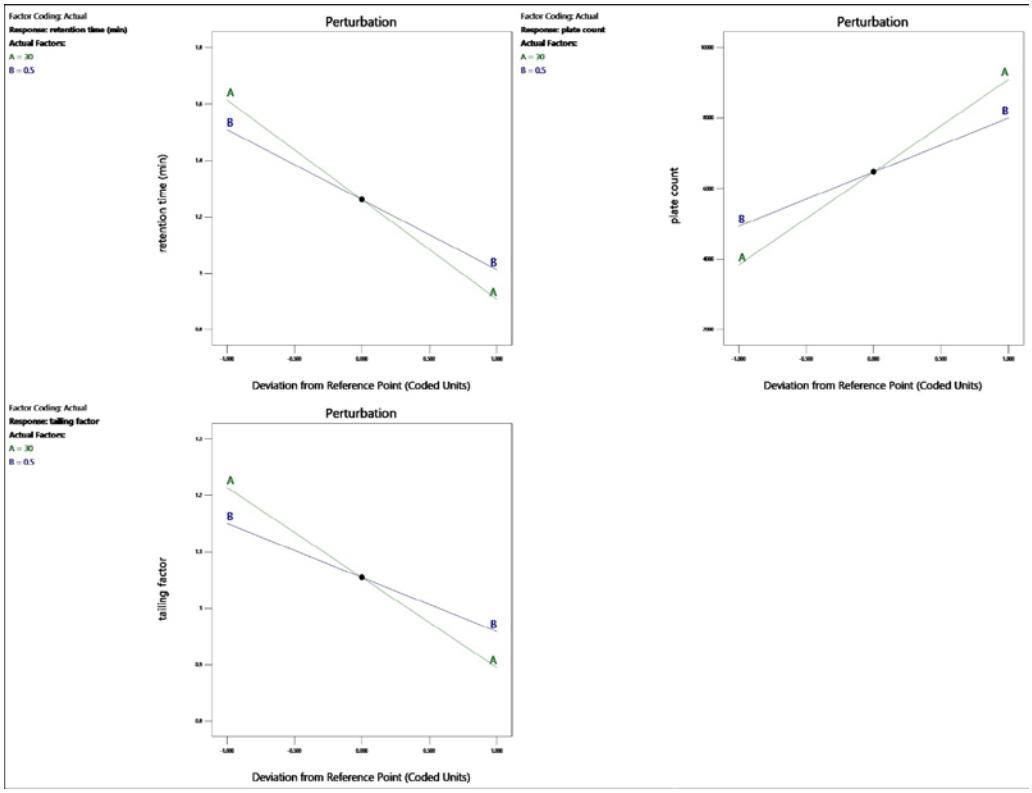

Figure 3a: Perturbation plot of Retention time, Plate count, and tailing factor

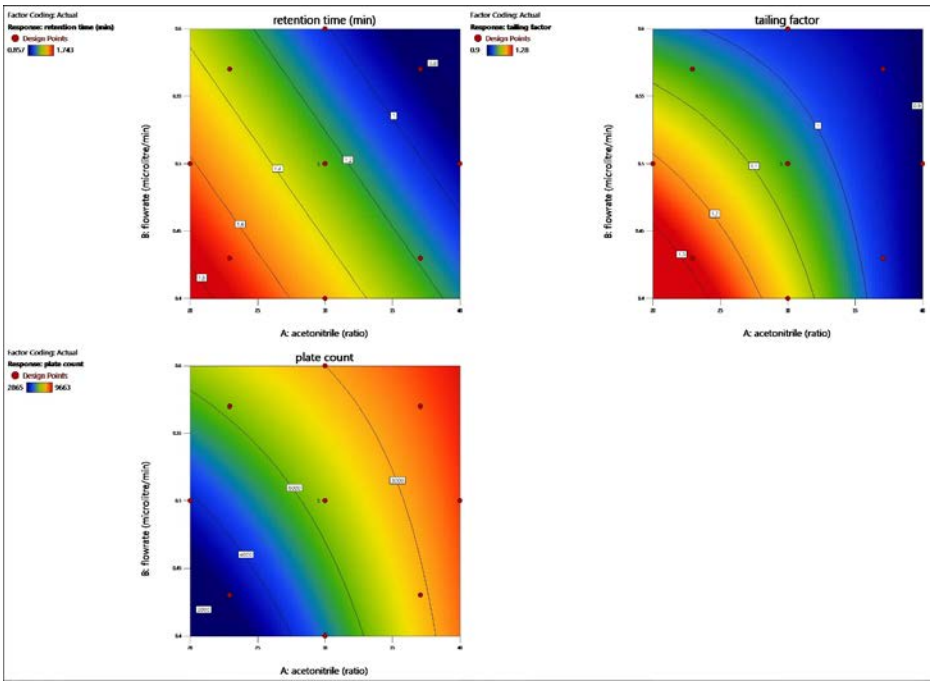

Figure 3b: 2D contour plot of retention time, tailing factor, and plate count

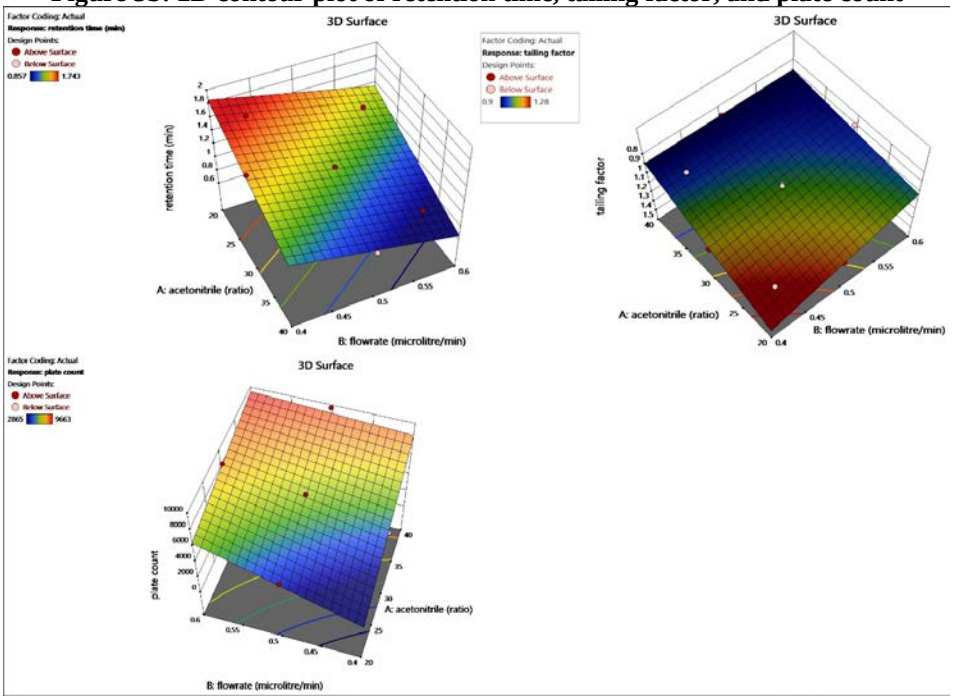

Figure 3c: 3D response plot of Retention time, Tailing factor, Plate count

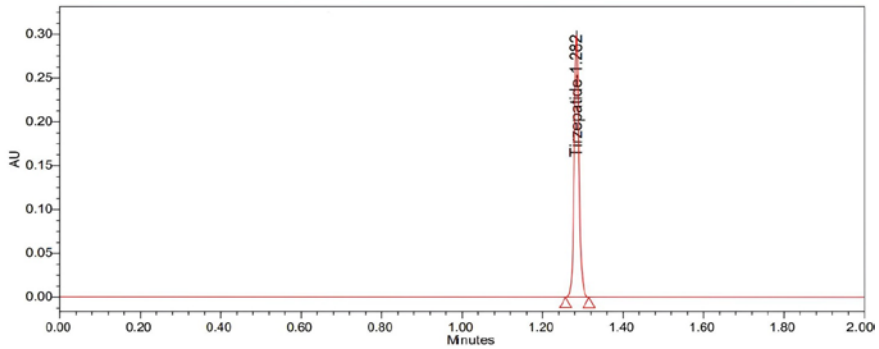

Figure 4: Final optimized chromatogram

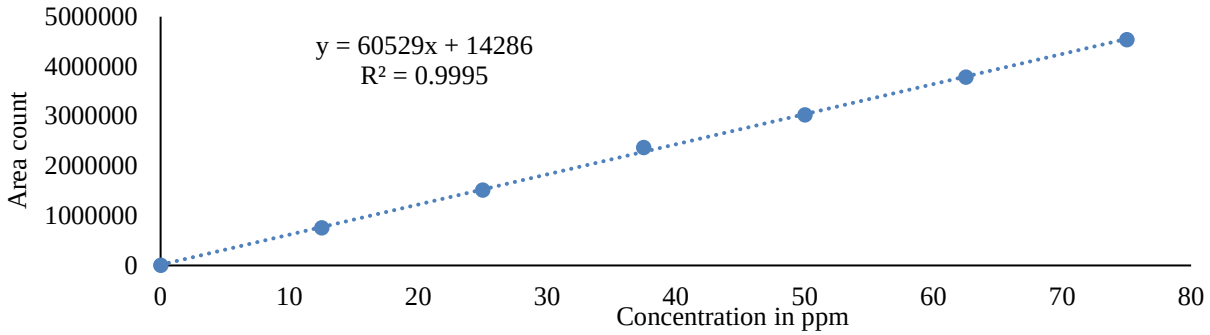

Figure 5: Linearity graph of Tirzepatide

Table 2: Results of System Suitability

Parameter	Tirzepatide
Theoretical plate count	6738
Tailing	1.04
Elution Time	1.282

Table 3: Linearity of the method

Tirzepatide		
S. No	**Concentration (µg/ml)**	**Area**
1	**12.50**	756613
2	**25.00**	1513226
3	**37.50**	2369838
4	**50.00**	3026451
5	**62.50**	3783064
6	**75.00**	4539677
r^2		0.9995
Slope		60529
Intercept		14286

Table 4: Results of method validation

s. no	Parameter	Result
1	**80 % Accuracy**	100.8 ± 0.44
2	**100 % Accuracy**	99.3± 0.27
3	**120 % Accuracy**	101.1± 0.319
4	**% Assay of formulation**	100.8 %
5	**Inter-day precision**	0.791
6	**Intra-day precision**	0.603
7	**LOD**	0.6 µg/ml
8	**LOQ**	2 µg/ml

Forced degradation studies

The stability and integrity of tirzepatide were analyzed by conducting forced degradation studies (Table 5) using the developed UPLC method.

Table 5: Forced degradation studies on Tirzepatide

Degradation condition	Area	%Label claim	% Degradation	Purity angle	Purity threshold	RT	Pass/Fail
Control	3029141	100	0	2.317	4.430	-	Pass
Acid	2991641	98.7	1.3	2.363	4.454	-	Pass
Alkali	2687053	88.7	11.3	2.382	4.479	1.399	Pass
Peroxide	2664137	87.9	12.1	2.391	4.446	1.632	Pass
Reduction	2967134	97.9	2.1	2.344	4.422	-	Pass
Thermal	2941871	97.1	2.9	2.338	4.417	-	Pass
Photo	2978134	98.3	1.7	2.323	4.431	-	Pass
Hydrolysis	2943022	97.1	2.9	2.314	4.428	-	Pass

In the presence of peroxide, tirzepatide exhibited the highest degradation percentage of 12.1%, with a degradation product peak observed at 1.632 min with a corresponding purity angle at 2.391, followed by the presence of sodium bisulphite with a degradation percentage of 11.3% with a degradation peak observed at 1.399 min with a corresponding purity angle at 2.382 (Figure 7). The lowest degradation percentage, 2.9% with a corresponding purity angle at 2.314, is observed in hydrolysis, followed by 2.9% with a corresponding purity angle at 2.338 in thermal degradation, 1.7% in photolysis with a corresponding purity angle at 2.323, and 1.3% in acid degradation with a corresponding purity angle at 2.363. The highest degradation percentage is observed in the presence of peroxide (Figure 6). This can be due to the composition of tirzepatide, which is a 39-amino-acid attached to a 20-carbon-long dicarboxylic fatty acid, as the acidic groups present are more prone to oxidative damage.

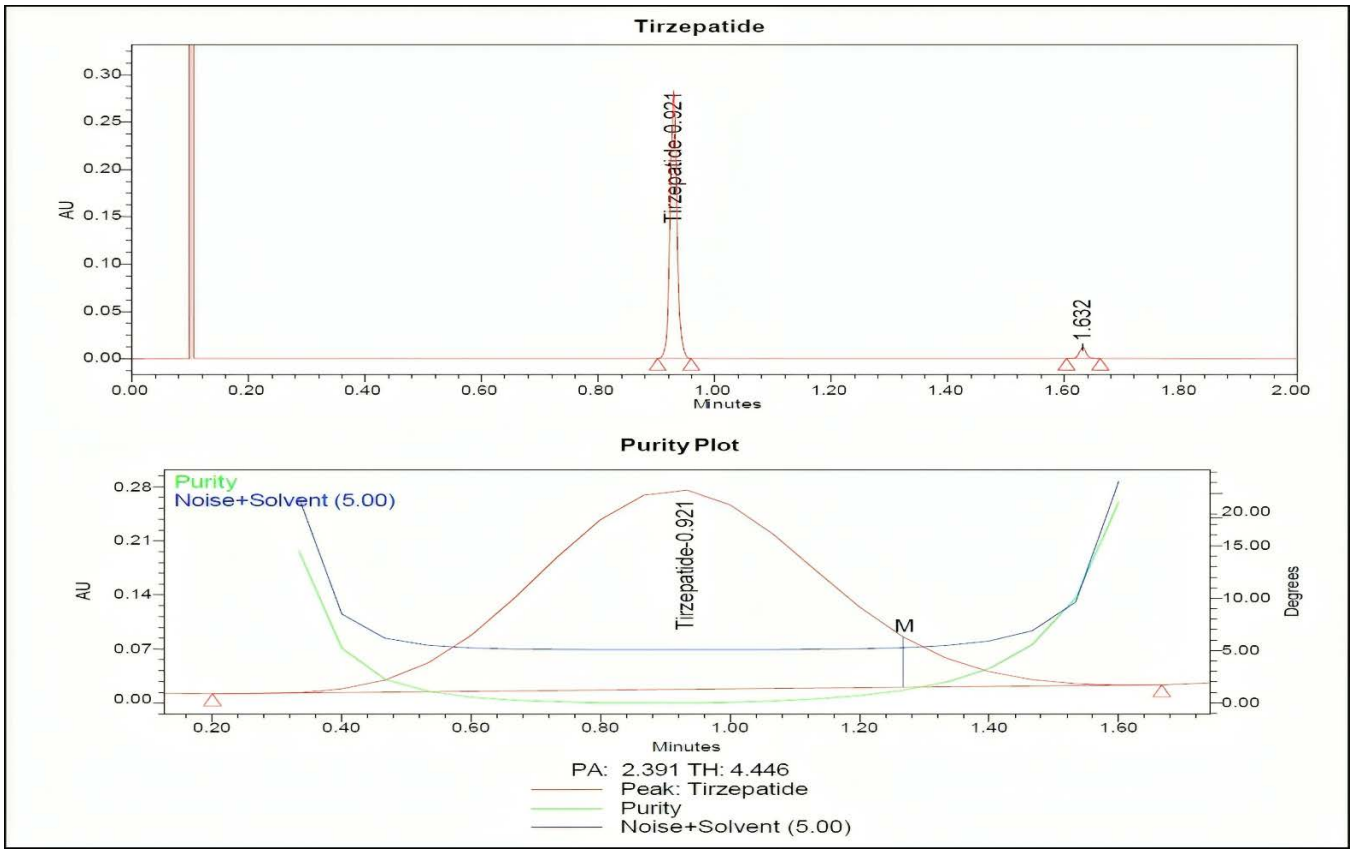

Figure 6: Peroxide degradation of Tirzepatide

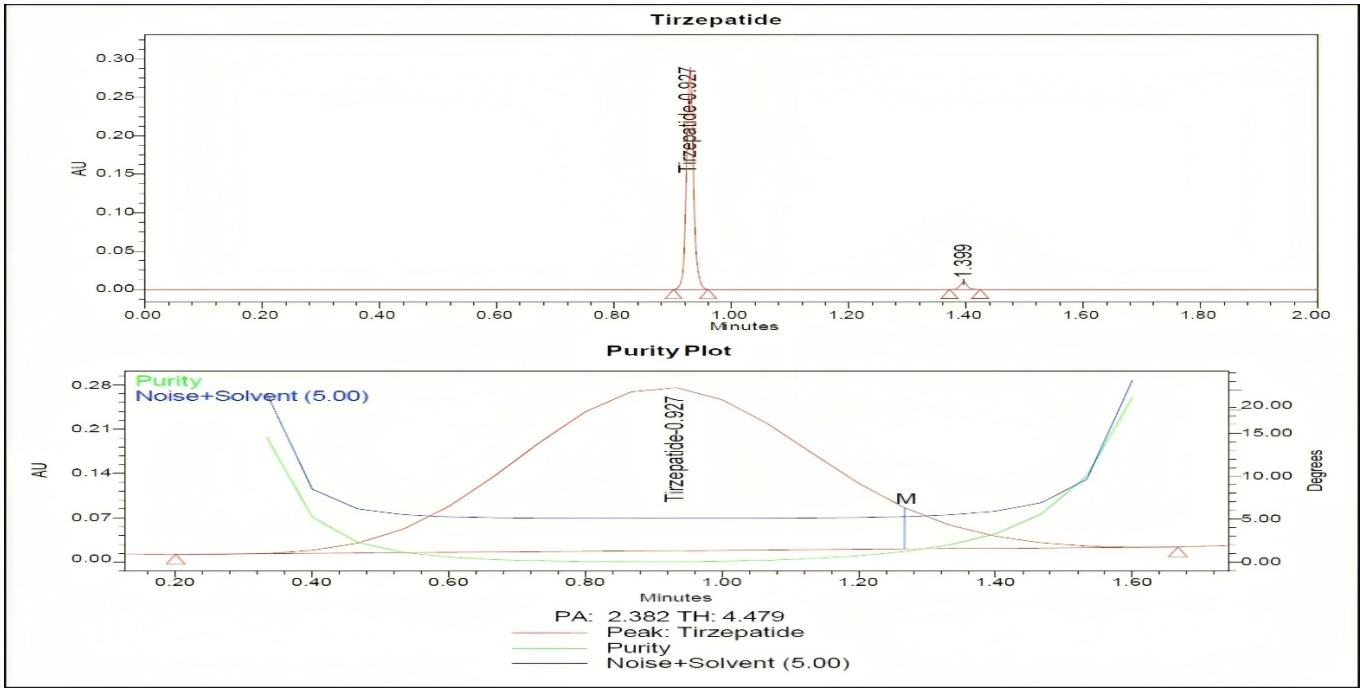

Figure 7: Alkali degradation of Tirzepatide

CONCLUSION

In this study, in accordance with ICH guidelines, a UPLC method was developed and validated. The DoE studies employed a systematic, scientific approach to optimizing methods to ensure reliability and accuracy. The systematic method optimization, like risk assessment, Factor screening, and response surface methodology, enabled the identification of Critical method variables, acetonitrile ratio, flow rate, and column temperature. The optimized method ensured a minimum retention time, more theoretical plates, and a lower tailing factor.

The method utilizes a Phenomenex C18 column (50 x 1.7 mm, 2.1 µm) at ambient temperature, with a mobile phase of acetonitrile and trifluoroacetic acid (37.07:62.93 v/v) at a flow rate of 0.57 mL/min, and a photodiode array detector at 264 nm, corresponding to the maximum absorbance of tirzepatide. The method achieves a short retention time of 1.282 min. The method validation results confirm linearity over 12.5-75 µg/mL (R^2 of 0.9995) with intraday and interday %RSD values of 0.603 and 0.791. The forced degradation studies reveal moderate degradation of tirzepatide under alkaline (11.3%) and peroxide (12.1%) conditions. The developed UPLC method aligns with ICH guidelines, ensuring robust quality control measures in the pharmaceutical industry to maintain batch-to-batch consistency of tirzepatide formulations.

FINANCIAL ASSISTANCE
NIL

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
M. Siva Kumar has designed the experiment, supervised the overall research work, revised the manuscript, and made necessary corrections, and Panthagada Sunitha has carried out the research work and drafted the preliminary version of the manuscript.

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