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Original Research Article

Method development and validation of tirzepatide in bulk and pharmaceutical dosage form by using RP-HPLC

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Abstract

Background: Tirzepatide is a novel dual glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist with significant clinical value in type 2 diabetes mellitus management. Analytical methods enabling accurate, robust, and stability-indicating quantification are essential for quality control of bulk and dosage forms.
Aim and Objective: To develop and validate a simple, precise, and stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for estimation of tirzepatide in bulk drug and pharmaceutical dosage form, in accordance with ICH Q2(R1) guidelines.
Materials and Methods: Chromatographic separation was achieved using an Agilent Eclipse XDB C18 column (150 × 4.6 mm, 3.5 µm) with an isocratic mobile phase of acetonitrile and 0.1% triethylamine buffer (pH 2.5, adjusted with orthophosphoric acid) in the ratio of 30:70 (v/v), at a flow rate of 1.0 mL/min. Detection was carried out at 234 nm with a 10 µL injection volume. Method validation parameters evaluated included specificity, linearity, accuracy, precision, robustness, and sensitivity (LOD/LOQ). Forced degradation studies under acid, alkali, oxidative, reductive, hydrolytic, photolytic, and thermal stress were performed to confirm stability-indicating capability.
Results: Tirzepatide eluted at 2.563 min with sharp and symmetrical peaks (tailing factor 1.15, plate count 10,524). The method showed excellent linearity over 25–150 µg/mL ($r^2 = 0.99979$), recoveries within 99.4–101.1% (mean 100.1%), and precision with %RSD < 2. Robustness testing under varied flow rates and mobile-phase ratios confirmed method resilience. Sensitivity studies yielded LOD and LOQ values of 0.60 µg/mL and 2.00 µg/mL, respectively. Forced degradation indicated maximum degradation under oxidative (11.6%) and reductive (12.6%) conditions, while thermal, photolytic, and hydrolytic conditions caused only minor changes; in all cases, peak purity remained intact.
Conclusion: The validated RP-HPLC method is accurate, precise, robust, and stability-indicating. Its short runtime, reproducibility, and simplicity make it highly suitable for routine quality control and stability testing of tirzepatide in pharmaceutical laboratories.

Keywords: Tirzepatide; RP-HPLC; method validation; stability-indicating assay; ICH Q2(R1).

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1. Introduction

Type 2 diabetes mellitus (T2DM) remains a major public health challenge, characterized by progressive β-cell dysfunction, insulin resistance, and a high burden of micro- and macrovascular complications.[1,2] Despite the availability of multiple therapeutic classes—metformin, insulin, sulfonylureas, thiazolidinediones, DPP-4 inhibitors, SGLT2 inhibitors, and GLP-1 receptor agonists—durable glycemic control often requires combination therapy that addresses complementary pathophysiologic defects. Incretin-based therapies have gained prominence because they improve glycemia with a favourable profile for body weight and hypoglycemia risk.[3] Within this class, tirzepatide is a first-in-class dual agonist of the glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptors. By co-activating both receptors, tirzepatide enhances glucose-dependent insulin secretion, suppresses glucagon in hyperglycemic states, delays gastric emptying, and reduces body weight—together yielding robust improvements in HbA1c and cardiometabolic parameters in adults with T2DM.[4-7]

From a pharmaceutical quality perspective, the growing clinical use of tirzepatide creates an imperative for simple, reliable, and transfer-ready analytical methods capable of assuring identity, strength, quality, and purity throughout the product lifecycle. While peptide and peptidomimetic drugs

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are frequently evaluated by high-resolution techniques (e.g., LC–MS, UPLC with advanced detectors, or orthogonal capillary methods), routine quality control (QC) in many manufacturing and regulatory settings still depends on conventional, robust, and cost-effective high-performance liquid chromatography (HPLC) with UV detection. Reversed-phase HPLC (RP-HPLC) is particularly attractive due to its broad column availability, manageable mobile-phase systems, straightforward system suitability metrics, and ease of transfer across laboratories.[7-13]

The present work addresses this need by describing the development and validation of an isocratic RP-HPLC method for the assay of tirzepatide in bulk drug and pharmaceutical dosage forms. Method validation followed ICH Q2 principles, encompassing specificity, linearity, range, accuracy, precision (system, method, and intermediate), robustness, and sensitivity (limits of detection and quantification). Forced-degradation studies under acid, base, oxidative, reductive, thermal, photolytic, and hydrolytic conditions were performed to confirm that the assay is stability-indicating and that the tirzepatide peak remains spectrally pure and chromatographically resolved from degradants under stress. In addition to meeting regulatory expectations, the method was designed to be time-efficient (≈5-min runtime), solvent-conserving, and easily transferable across standard HPLC platforms equipped with PDA or UV detectors.[13-17]

2. Materials and Methods

2.1. Chemicals and reagents

Analytical work employed HPLC-grade acetonitrile (Merck), triethylamine (Merck), and orthophosphoric acid (Merck). Purified water was produced in-house using a Milli-Q system. Unless otherwise stated, acetonitrile served as the diluent for standards and samples. All chemicals were used as received without further purification.

2.2. Instruments

Chromatographic analyses were performed on a Waters Alliance HPLC system equipped with a photodiode array detector. Ancillary instrumentation comprised a Eutech pH 700 meter for buffer pH adjustment, a Sartorius BSA224S-CW analytical balance for gravimetry, and a Shimadzu UV-1700 UV/Vis spectrophotometer for spectral measurements. Class-A borosilicate glassware (borosil) was used for all volumetric operations, and solutions were degassed and aided in dissolution using a unichrome UCA-701 ultrasonicator. An isocratic pump (Waters) provided constant flow during all chromatographic runs.

2.3. Determination of λmax

The working wavelength for quantification was established by scanning tirzepatide solutions between 200 and 400nm using the PDA detector. Solutions were prepared in a mixture of acetonitrile and 0.1% triethylamine adjusted to pH 2.5 with orthophosphoric acid (30:70, v/v), with the same mixture as the blank. The spectrum exhibited a distinct isosbestic point at 234 nm; this wavelength was therefore selected for detection in all subsequent HPLC measurements (**Figure 1**, UV scan highlighting 234nm).

2.4. Method development and final chromatographic conditions

Method development entailed screening combinations of stationary phases and mobile-phase compositions to balance retention, efficiency, and peak symmetry while maintaining a short run time. The optimized method used an Agilent Eclipse XDB C18 column (150 × 4.6mm, 3.5µm). The mobile phase consisted of acetonitrile and 0.1% triethylamine buffer at pH 2.5 (adjusted with orthophosphoric acid) in a 30:70 (v/v) ratio, delivered isocratically at 1.0 mL/min. Detection was carried out at 234 nm with an injection volume of 10µL and a total runtime of 5 min. Under these conditions tirzepatide eluted at 2.563 min with a mean peak area of approximately 2.77 × 10^6, a tailing factor of 1.15, and theoretical plates not less than 2000.

2.5. Preparation of solutions

For buffer preparation, 1 mL of triethylamine was dissolved in 1L of HPLC water, the pH was adjusted to 2.5 using orthophosphoric acid, and the solution was filtered through a 0.45µm membrane. The mobile phase was prepared by mixing acetonitrile with the pH-adjusted 0.1% triethylamine buffer in a 30:70 (v/v) proportion, followed by filtration and degassing through a 0.45µm membrane. The standard stock solution was prepared by dissolving 10mg of tirzepatide working standard in acetonitrile and making up the volume to 10mL (1000µg/mL). A 100µg/mL working standard was obtained by diluting 1 mL of this stock to 10 mL with diluent. The sample solution, prepared analogously to the standard, was sonicated for 30min to ensure complete dissolution; a 100µg/mL working sample solution was then obtained by a further 10-fold dilution with diluent and filtered through a 0.45µm syringe filter prior to injection.

2.6. System suitability

Prior to analysis, system suitability was verified in accordance with accepted chromatographic practice. The criteria included a tailing factor not more than 2.0 for the analyte peak, theoretical plates not less than 2000 for the column under the method conditions, and a relative standard deviation of peak areas not exceeding 2.0% for six replicate injections of the standard solution.

2.7. Assay procedure and calculation

Analytical runs comprised sequential injections of the blank, the standard, and the test sample (10µL each). Chromatograms were recorded at 234nm, and the tirzepatide peak area was used for quantification. The percentage assay of tirzepatide in the sample was calculated

$$\% \text{ Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{DT}{WT} \times \frac{(\text{Average weight})}{(\text{Label Claim})} \times \frac{P}{100} \times 100$$

Where, AT= average area counts of test (sample) preparation; AS= average area counts of standard preparation;

WS= Weight of working standard taken in mg; DS=Dilution of working standard in ml; DT= Dilution of test (sample) in ml;

WT= Weight of test (sample) taken in mg; P= Percentage purity of working standard; LC= Label Claim mg/ml.

2.8. Method validation (ICH Q2)

Validation followed ICH Q2 principles. Specificity was demonstrated by the absence of interfering responses at the analyte retention time in blank and placebo chromatograms, as well as by the purity of the analyte peak in standard and sample solutions. Linearity was assessed over 25–150 µg/mL using six concentration levels; calibration plots of peak area versus concentration afforded a correlation coefficient not less than 0.999. The validated analytical range was consequently established across this interval, supported by accuracy and precision outcomes. Accuracy was evaluated by recovery studies at approximately 80%, 100%, and 120% of the target assay concentration (about 180, 200, and 220µg/mL, respectively), with recoveries expected within 98.0–102.0% at each level.

Precision encompassed system precision (six injections of the standard), method precision (six independent sample preparations), and intermediate precision (different days/analysts/instruments as applicable), with percent RSD limits not exceeding 2.0% for peak areas and assay results. Method robustness was examined by deliberately varying the flow rate to 0.9 and 1.1 mL/min and by adjusting the organic component of the mobile phase by ±2% (absolute) relative to the optimized 30:70 composition; system suitability and assay values were required to remain within predefined limits under these perturbations. Sensitivity was established by estimating the limit of detection and limit of quantification using the ICH slope-and-standard-deviation approach, yielding LOD and LOQ values of 0.60 µg/mL and 2.00 µg/mL, respectively, corresponding to signal-to-noise ratios of approximately 3 and 10.

2.9. Forced degradation (stability-indicating capability)

To demonstrate the stability-indicating nature of the method, tirzepatide was subjected to acid and base hydrolysis, oxidative and reductive stress, neutral hydrolysis, photolysis, and thermal degradation under conditions aligned with ICH recommendations. After stress exposure, solutions were neutralized where appropriate, diluted to within the validated working range, filtered through 0.45µm membranes, and analysed using the optimized method. Peak purity, resolution from degradation products, and the extent of degradation (percent loss relative to control) were evaluated. Across all stressors, the tirzepatide peak remained spectrally pure and chromatographically resolved from its degradation products, confirming that the assay conditions are stability-indicating.

3. Results

3.1. Optimization of chromatographic conditions

During method development, several chromatographic parameters including the stationary phase, mobile phase composition, and detection wavelength were systematically varied. The optimized conditions were achieved using an Agilent Eclipse XDB C18 column (150 × 4.6 mm, 3.5 µm) with a mobile phase consisting of acetonitrile and 0.1% triethylamine buffer at pH 2.5, adjusted with orthophosphoric acid, in the ratio of 30:70 (v/v) (**Figure 1**). The flow rate was maintained at 1.0 mL/min, the injection volume was 10µL, and detection was carried out at 234 nm. Under these conditions, tirzepatide eluted with a sharp, symmetrical peak at a retention time of 2.563 minutes. The peak area obtained was approximately 2,766,102, with a tailing factor of 1.15 and a theoretical plate count of 10,524.

All the system suitability parameters were well within the limits specified by ICH guidelines, which require a tailing factor less than 2.0 and theoretical plates greater than 2000. The observed results confirm that the optimized chromatographic conditions are appropriate for the analysis of tirzepatide and suitable for subsequent method validation.

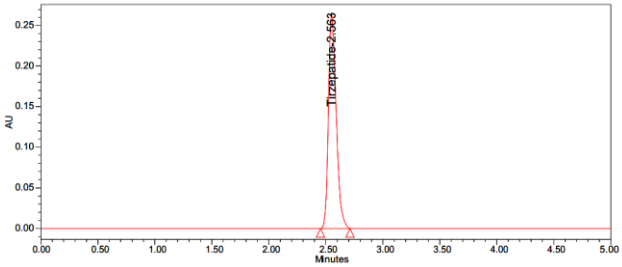

Figure 1: Optimized chromatogram of tirzepatide under validated chromatographic conditions

3.2. System suitability

System suitability testing confirmed that all parameters were within the acceptance limits recommended by ICH guidelines. In **Table 1** the retention time of tirzepatide was 2.563 min, the plate count was 10,524, the tailing factor was 1.15, and the %RSD of replicate injections was 0.15, all well within limits (tailing factor < 2, plates > 2000, %RSD < 2%). These results demonstrated the suitability of the system for further validation studies.

3.3. Specificity

Chromatograms of blank and placebo solutions showed no interference at the retention time of tirzepatide (2.563 min), confirming the specificity of the method.

3.4. Precision

Precision was evaluated through system precision, method precision, and intermediate precision. In **Figure 2** system precision was established by injecting six replicates of the standard solution at 100µg/mL. The %RSD of peak areas was 0.15, confirming reproducibility. Method precision (repeatability) was determined by six replicate injections of the sample solution, yielding %RSD = 0.78. Intermediate precision, evaluated across different days, showed inter-day %RSD values between 0.53 and 0.64. All results complied with the ICH acceptance criterion of %RSD ≤ 2.

3.5. Linearity

In table **Table 3** Linearity was assessed in the range of 25–150µg/mL. The calibration curve was linear with a regression equation of y = 27150.53x + 17857.36 and correlation coefficient (r^2) of 0.99979. These results indicate excellent proportionality between peak area and analyte concentration.(**Figure 2**)

3.6. Accuracy

In **Table 4** accuracy was determined by recovery studies at 80%, 100%, and 120% of target concentration. Recoveries ranged between 99.4% and 101.1%, with a mean recovery of 100.1%.

3.7. Assay

The assay of tirzepatide in pharmaceutical dosage form yielded 99.4% of the label claim, indicating the accuracy and applicability of the method for routine analysis.

3.8. Robustness

In **Table 5** Robustness was confirmed by deliberate variations in flow rate (0.9–1.1mL/min) and organic composition of the mobile phase (±3%). None of the variations significantly impacted retention time, peak area, tailing factor, or theoretical plate count, confirming robustness of the method.

3.9. Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) for tirzepatide were found to be 0.60 µg/mL and 2.00 µg/mL, respectively, corresponding to signal-to-noise ratios of approximately 3 and 10.

3.10. Forced Degradation Studies

Tirzepatide was subjected to various stress conditions in line with ICH guidelines. In **Table 6** the drug was most susceptible to oxidative (11.6% degradation) and reductive (12.6%) conditions, while it remained relatively stable under thermal, photolytic, and hydrolytic stress. Importantly, peak purity indices confirmed that the tirzepatide peak was spectrally pure under all stress conditions, demonstrating the stability-indicating nature of the developed method.(**Figure 3**)

Table 1: System suitability parameters for Tirzepatide

Parameter	Value
Retention time	2.563 min
Plate count	10,524
Tailing factor	1.15
%RSD	0.15

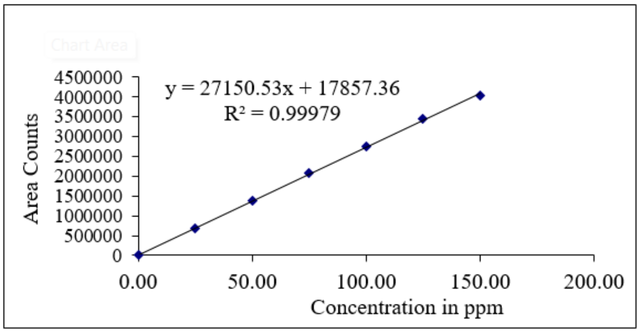

Figure 2: Calibration curve of tirzepatide at 234 nm.

Table 2: System precision results of tirzepatide

S. No.	Conc. (µg/mL)	Area
1	100	2,766,102
2	100	2,771,544
3	100	2,774,845
4	100	2,765,452
5	100	2,763,652
6	100	2,769,455
Mean	—	2,768,508
SD	—	4,218.54
%RSD	—	0.15

Table 3: Linearity results for tirzepatide

Conc. (µg/mL)	Peak area
25	689,478
50	1,378,955
75	2,068,433
100	2,757,910
125	3,447,388
150	4,036,865

Table 4: Accuracy results of tirzepatide by HPLC method

Level	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean % recovery
80%	4,996,463	18	18.047	100.3	100.1
	4,974,284	18	17.967	99.8	
	4,991,312	18	18.029	100.2	
100%	5,516,102	20	19.924	99.6	100.4
	5,597,618	20	20.219	101.1	
	5,557,284	20	20.073	100.4	
120%	6,082,031	22	21.969	99.9	99.7
	6,084,570	22	21.978	99.9	
	6,056,456	22	21.876	99.4	

Table 5: Robustness results of tirzepatide by HPLC

Parameter	Condition	Retention time (min)	Peak area	Tailing	Plate count	%RSD
Flow rate	0.9 mL/min	2.682	2,632,279	1.20	10,413	0.83
	1.0 mL/min	2.563	2,766,102	1.15	10,524	0.15
	1.1 mL/min	2.364	2,970,105	1.12	10,719	0.44
Organic ratio	27:73	2.844	2,493,129	1.16	10,266	0.47
	30:70	2.568	2,771,544	1.11	10,557	0.15
	33:67	2.199	3,109,870	1.07	10,871	0.85

Table 6: Forced degradation results for tirzepatide

Condition	Area	% Assay	% Degradation	Purity angle	Purity threshold
Control	2,768,714	100.0	0.0	1.758	4.690
Acid	2,632,365	95.1	4.9	1.763	4.628
Alkali	2,509,742	90.7	9.3	1.779	4.637
Peroxide	2,447,678	88.4	11.6	1.741	4.651
Reduction	2,420,341	87.4	12.6	1.702	4.646
Thermal	2,705,740	97.7	2.3	1.789	4.628
Photolytic	2,694,765	97.3	2.7	1.745	4.691
Hydrolysis	2,684,633	97.0	3.0	1.720	4.602

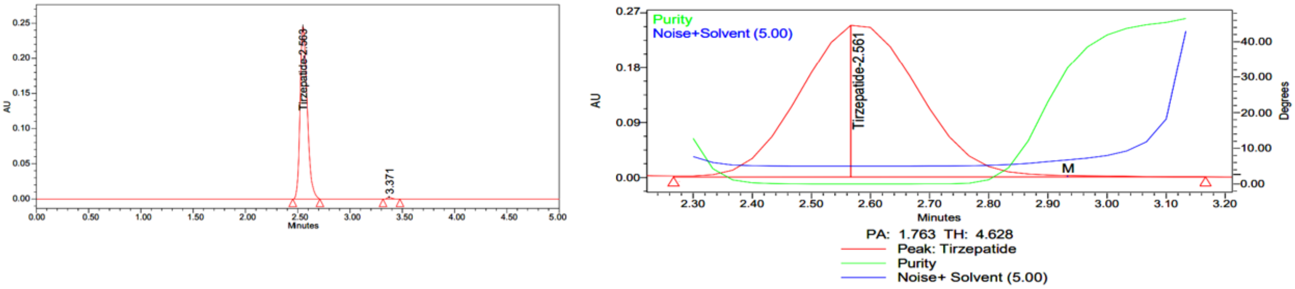

Figure 3: Representative chromatograms of stressed and control samples showing stability-indicating separation.

4. Discussion

This work established and validated a rapid, isocratic RP-HPLC method for quantitative estimation of tirzepatide in bulk and dosage forms, emphasizing routine QC applicability. Chromatographic optimization converged on an Agilent Eclipse XDB C18 (150 × 4.6mm, 3.5µm) operated with acetonitrile–0.1% triethylamine buffer at pH 2.5 (30:70, v/v) and detection at 234 nm. Under these conditions, tirzepatide eluted at 2.563 min with excellent peak symmetry (tailing factor 1.15) and high efficiency (plate count 10,524), enabling a total runtime of 5 min. The use of an acidic TEA buffer likely mitigated secondary interactions of the peptide with residual silanol sites and metallic surfaces, thereby improving peak shape and retention stability without resorting to gradient elution or ion-pair reagents that can complicate transferability.[18]

System suitability (%RSD 0.15 for peak areas; plates > 2000; tailing < 2) confirmed instrument readiness and method ruggedness. Linearity across 25–150 µg/mL ($r^2 = 0.99979$) demonstrated proportionality between response and concentration within a range that comfortably brackets the target assay level. Accuracy by recovery (80/100/120%) fell within 99.4–101.1% (mean ≈ 100.1%), and precision metrics—all ≤ 0.78% for system/method and ≤ 0.64% inter-day—underscore repeatability and intermediate precision suitable for release and stability testing. Sensitivity (LOD 0.60 µg/mL; LOQ 2.00 µg/mL) is adequate for low-level monitoring during robustness checks and supports confident quantitation around the assay level.

Robustness studies indicated that moderate perturbations in flow (±0.1 mL/min) and organic content (±3% absolute) did not adversely affect key performance attributes (retention, area, symmetry, efficiency), with all %RSD values ≤ 0.85. This tolerance to common day-to-day variations supports method sustainability in QC environments and across laboratories.[19]

Forced degradation established the method's stability-indicating capability. Tirzepatide exhibited the greatest susceptibility under oxidative (≈11.6%) and reductive (≈12.6%) stress, with comparatively minor losses under thermal, photolytic, and hydrolytic conditions (≈2–3%). Across all stressors, peak purity metrics remained acceptable (purity angle < purity threshold), and no interfering degradant co-eluted with the analyte peak, confirming chromatographic resolution of degradants at the working λ and conditions. These observations are consistent with peptide-like molecules in which oxidation and reduction can drive side-chain perturbations, whereas strongly acidic conditions (pH 2.5) during analysis help maintain a consistent chromatographic environment.

5. Conclusion

A fast, accurate, precise, and robust isocratic RP-HPLC method was developed and validated for Tirzepatide in bulk and pharmaceutical dosage forms. Using a C18 column with acetonitrile–0.1% TEA buffer (pH 2.5, 30:70, v/v) and UV detection at 234 nm, the method achieved a 2.563-min retention time, excellent peak symmetry (tailing 1.15), high efficiency (N = 10,524), and outstanding precision (system %RSD 0.15; method %RSD 0.78; inter-day ≤ 0.64). Linearity (25–150µg/mL, $r^2 = 0.99979$), mean recovery near 100%, and sensitivity (LOD 0.60 µg/mL; LOQ 2.00µg/mL) met ICH criteria, while forced-degradation studies confirmed the method's stability-indicating nature with resolved, pure analyte peaks under all stress conditions. The procedure's simplicity, short runtime, and robustness make it well-suited for routine QC release testing and stability studies. Future extensions can include MS-compatible variants for degradant identification, controlled-temperature robustness assessments, and broader matrix evaluations to support lifecycle management and inter-laboratory transfer.

6. Conflict of Interest

None.

7. Source of Funding

None.

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