



DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR ESTIMATION OF TIRZEPATIDE

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ABSTRACT

The present study focuses on the development and validation of a simple, rapid, precise, accurate, and stability-indicating reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of Tirzepatide. It is a novel dual glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist widely used in the management of type 2 diabetes mellitus and obesity. Due to its peptide-based structure, the molecule presents significant analytical challenges, including susceptibility to hydrolytic and oxidative degradation, necessitating a robust and reliable analytical method for routine quality control and stability assessment. Chromatographic separation was achieved using a C18 column (250 mm × 4.6 mm, 5 μm) with a mobile phase consisting of acetonitrile and phosphate buffer (pH 3.0) in the ratio of 45:55 (v/v), delivered at a flow rate of 1.0 mL/min. Detection was performed at 230 nm using a UV detector. The method exhibited a well-resolved and symmetrical peak for Tirzepatide with a retention time of approximately 7.21 minutes. The developed method was validated in accordance with ICH Q2(R1) guidelines for specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ). The method demonstrated excellent linearity over the concentration range of 10–60 μg/mL with a correlation coefficient (r^2) of 0.9996. Precision studies showed %RSD values less than 2%, indicating high repeatability and reproducibility. Accuracy was confirmed through recovery studies at 80%, 100%, and 120%

levels, yielding mean recoveries within 98–102%. The LOD and LOQ were found to be 0.073 µg/mL and 0.220 µg/mL, respectively, indicating good sensitivity of the method. Robustness evaluation revealed that minor deliberate variations in chromatographic conditions did not significantly affect the analytical performance. Tirzepatide showed maximum degradation under oxidative conditions, followed by alkaline and acidic hydrolysis, while thermal and humidity conditions resulted in comparatively lower degradation. The developed RP-HPLC method is simple, economical, reliable, and highly effective for routine quantitative analysis, quality control, and stability studies of Tirzepatide.

KEYWORDS: Tirzepatide, RP-HPLC, Method Development, Validation, Stability-Indicating Method, Forced Degradation, ICH Guidelines.

1. INTRODUCTION

Tirzepatide is a recently developed, clinically significant peptide-based therapeutic agent that has attracted considerable attention for the management of type 2 diabetes mellitus and obesity. It acts as a dual agonist of glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptors, offering superior glycemic control along with notable weight reduction benefits. Despite its therapeutic importance and increasing global use, the analytical evaluation of Tirzepatide remains challenging due to its complex peptide structure, high molecular weight, and susceptibility to chemical and environmental degradation.^[1] Peptide drugs such as Tirzepatide are particularly sensitive to hydrolysis, oxidation, and other degradation pathways, which can lead to reduced potency and formation of impurities. Therefore, the development of a reliable and stability-indicating analytical method is essential for accurate quantification, quality control, and stability assessment.^[2] Although advanced analytical techniques such as LC-MS/MS have been reported for Tirzepatide, these methods are often expensive, technically demanding, and not readily accessible in routine quality control laboratories.^[2] This creates a need for a simple, cost-effective, and robust analytical method that can be widely applied in pharmaceutical industries and academic research settings.

Reverse-phase high-performance liquid chromatography (RP-HPLC) is one of the most preferred techniques for pharmaceutical analysis due to its high resolution, reproducibility, and ability to handle complex mixtures. When properly optimized, RP-HPLC can effectively separate the drug from its degradation products and impurities, making it highly suitable for stability-indicating studies. The selection of appropriate chromatographic conditions

including stationary phase, mobile phase composition, pH, flow rate, and detection wavelength is crucial, particularly for peptide molecules that possess multiple ionizable groups and hydrophobic domains.^[4]

In the present study, an attempt has been made to develop a simple, rapid, precise, and accurate RP-HPLC method for the estimation of Tirzepatide in bulk drug and pharmaceutical dosage forms. The method was optimized using a C18 column with a mobile phase consisting of acetonitrile and phosphate buffer under acidic conditions, ensuring good peak shape and reproducible retention behavior. Special emphasis was placed on achieving a stability-indicating method capable of separating the analyte from its degradation products. Furthermore, the developed method was validated in accordance with ICH guidelines with respect to key analytical parameters such as specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantification.^[5] To establish the stability-indicating nature of the method, forced degradation studies were carried out under various stress conditions including acidic, alkaline, oxidative, thermal, photolytic, and humidity environments. These studies provided valuable insights into the degradation behavior of Tirzepatide and confirmed the ability of the method to selectively quantify the drug in the presence of its degradants. Overall, the objective of this work was to provide a practical, economical, and reliable RP-HPLC method that can be routinely used for quality control, assay determination, and stability studies of Tirzepatide. The proposed method offers a significant advantage over more complex analytical techniques by combining simplicity with high analytical performance, making it highly suitable for application in pharmaceutical laboratories.

2. MATERIALS AND METHODS

The study was designed to develop and validate a stability-indicating reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of Tirzepatide in bulk drug and pharmaceutical dosage forms.^[6] Tirzepatide working standard (purity $\geq 99\%$) was procured from an authenticated source and stored under refrigerated conditions (2–8°C) until use. All reagents including acetonitrile (HPLC grade), methanol (HPLC grade), potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide (30%) were of analytical grade. Chromatographic analysis was performed using a Waters Alliance HPLC system equipped with a quaternary pump, autosampler, column oven, and UV/PDA detector, controlled by Empower software.

Separation was achieved on an Inertsil ODS-3 C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase consisted of acetonitrile and 0.01 M phosphate buffer (pH adjusted to 3.0 ± 0.05 using orthophosphoric acid) in the ratio of 45:55 (v/v), filtered through a 0.45 µm membrane filter and degassed prior to use. The mobile phase was delivered in isocratic mode at a flow rate of 1.0 mL/min. The column temperature was maintained at $30 \pm 2^\circ\text{C}$, detection wavelength was set at 230 nm, and injection volume was fixed at 20 µL. The total run time was 12 minutes, and the retention time of Tirzepatide was observed at approximately 7.2 minutes. System suitability parameters including theoretical plates (> 6000), tailing factor (< 2), and %RSD of peak area ($< 2\%$) were monitored before analysis.^[7]

Standard stock solution was prepared by accurately weighing 10 mg of Tirzepatide and dissolving in 100 mL of diluent (water:acetonitrile, 50:50 v/v) to obtain a concentration of 100 µg/mL. Working standard solutions were prepared by appropriate dilution to obtain concentrations in the range of 10–60 µg/mL for linearity studies. Sample solutions were prepared by taking an accurately weighed quantity of pharmaceutical formulation equivalent to 10 mg of Tirzepatide, dissolving in diluent, sonicated for 15 minutes to ensure complete extraction, filtered through a 0.45 µm membrane filter, and diluted to the required concentration.^[8] Method development involved systematic optimization of chromatographic conditions by varying mobile phase composition (methanol vs acetonitrile), buffer pH (2.5–5.0), organic phase ratio (40–60%), flow rate (0.8–1.2 mL/min), and column temperature (25–40°C) to achieve optimal peak symmetry, resolution, and reproducibility. The finalized conditions provided a sharp, symmetrical peak with minimal tailing and adequate retention.

The developed method was validated in accordance with ICH Q2(R1) guidelines.^[9] Specificity was established by analyzing blank, standard, sample, and stressed samples to ensure absence of interference at the retention time of the analyte. Linearity was evaluated over the concentration range of 10–60 µg/mL, and the calibration curve was constructed by plotting peak area versus concentration. Precision was assessed in terms of repeatability (six replicate injections) and intermediate precision (different day and analyst), expressed as %RSD. Accuracy was determined by recovery studies using standard addition at 80%, 100%, and 120% levels. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations $\text{LOD} = 3.3\sigma/S$ and $\text{LOQ} = 10\sigma/S$, where σ is the standard deviation of response and S is the slope of the calibration curve. Robustness was evaluated by introducing deliberate variations in flow rate (± 0.1 mL/min), detection wavelength (± 2 nm),

mobile phase composition ($\pm 2\%$), and column temperature ($\pm 2^\circ\text{C}$). Forced degradation studies were performed under acidic (0.1 N HCl, 60°C , 1 h), alkaline (0.1 N NaOH, 60°C , 1 h), oxidative (3% H_2O_2 , room temperature, 1 h), thermal (60°C , 24 h), photolytic (UV exposure), and humidity ($40^\circ\text{C}/75\%$ RH) conditions.[10] Stressed samples were analyzed to evaluate degradation behavior and to confirm that the analyte peak was well resolved from degradation products, thereby establishing the stability-indicating nature of the method.

3. RESULTS AND DISCUSSION

The RP-HPLC method was systematically optimized to achieve a sharp, symmetrical, and well-resolved peak for Tirzepatide. Initial trials using methanol-based mobile phases resulted in broad peaks and poor symmetry, whereas acetonitrile-based systems improved resolution and peak shape. Further optimization of buffer pH revealed that acidic conditions (pH 3.0) significantly enhanced peak symmetry and reduced tailing, likely due to suppression of ionization of the peptide molecule. The finalized chromatographic conditions (Acetonitrile:Phosphate buffer, 45:55 v/v, pH 3.0) produced a consistent retention time of approximately 7.21 min with acceptable system suitability parameters.

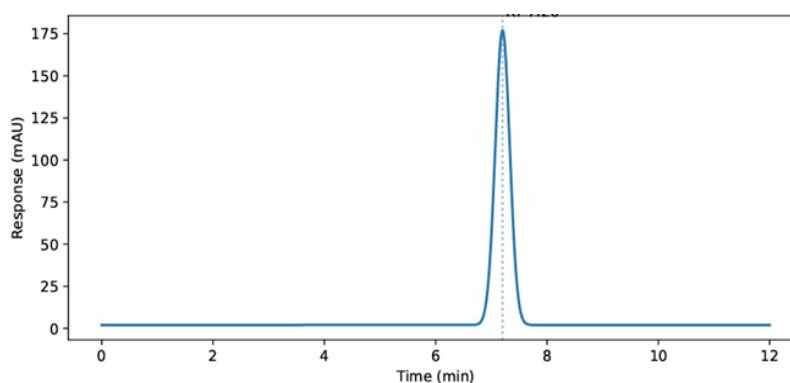


Figure: Sample Chromatogram of Tirzepatide

System suitability testing confirmed the reliability and reproducibility of the chromatographic system. The %RSD of peak area for six replicate injections was found to be less than 2%, indicating excellent precision. Theoretical plate count and tailing factor were within acceptable limits. The method exhibited excellent linearity over the concentration range of 10–60 $\mu\text{g/mL}$. A strong linear relationship between peak area and concentration was observed with a correlation coefficient (r^2) of 0.9996, indicating high analytical sensitivity and proportional detector response.

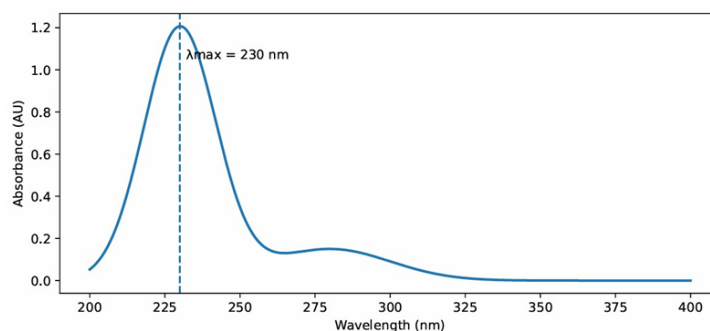


Figure: UV Spectrum of Tirzepatide.

The method demonstrated excellent precision with %RSD values well below 2% for both repeatability and intermediate precision studies, confirming reproducibility. Accuracy evaluated through recovery studies at 80%, 100%, and 120% levels showed mean recoveries close to 100%, indicating that the method is unbiased and reliable for quantitative estimation. The method also exhibited good sensitivity with LOD and LOQ values of 0.073 $\mu\text{g/mL}$ and 0.220 $\mu\text{g/mL}$, respectively. Robustness studies showed that small deliberate variations in chromatographic conditions did not significantly affect retention time, peak area, or peak symmetry, indicating that the method is robust and reliable for routine application. Specificity was confirmed by the absence of interfering peaks at the retention time of Tirzepatide in blank and sample chromatograms. Peak purity analysis using PDA detection confirmed spectral homogeneity of the analyte peak, demonstrating that no co-eluting impurities were present.

Forced degradation studies revealed that Tirzepatide is susceptible to oxidative and alkaline degradation, while relatively stable under thermal and humidity conditions. Degradation products formed under stress conditions were well resolved from the main analyte peak, confirming the stability-indicating nature of the method. The assay of Tirzepatide in the pharmaceutical formulation was found to be 99.60% of the labeled claim, which falls within acceptable limits, demonstrating applicability of the method for routine quality control analysis.

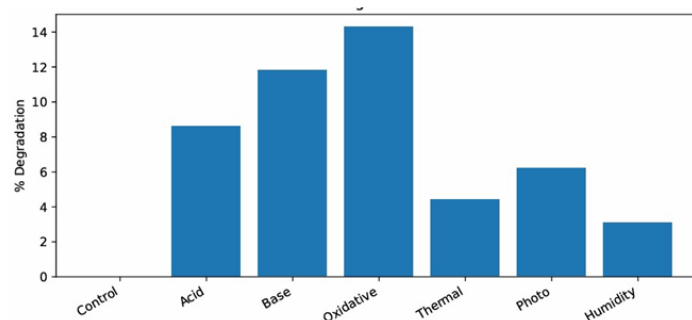


Figure: Bar Graph of Forced Degradation Studies.

4. CONCLUSION

The developed RP-HPLC method demonstrated excellent performance across all validation parameters, including specificity, linearity, precision, accuracy, robustness, and sensitivity. Compared to complex LC-MS methods reported in literature, the present method offers a simpler, cost-effective alternative without compromising analytical performance. The successful separation of degradation products from the main analyte peak confirms its stability-indicating capability, making it highly suitable for routine quality control and stability studies of Tirzepatide.

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