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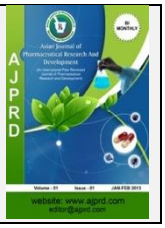

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Review Article

# Advancements in RP-HPLC Methodology for Tirzepatide Estimation in Human Plasma: Development, Validation, and Applications

**Vaishnavi N.Bitne*[1], Dr.Vinayak M. Gaware[2]**

[1]Department of Quality Assurance, PRES'S College of Pharmacy (For Women), Chincholi, Nashik
[2]Department of Quality Assurance, PRES'S College of Pharmacy (For Women), Chincholi, Nashik

## A B S T R A C T

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a technique that separates molecules by polarity. It's commonly used in pharmaceutical development and manufacturing, as well as in proteomic research. The goal of the proposed method is to develop and validate a new green and simple liquid chromatographic system for simultaneously quantifying the novel drug terzepatide. A simple, specific, precise and accurate method for quantification of tirzepatide was developed and validated in human plasma and was applied for pharmacokinetics to determination the concentration of tirzepatide in human low dose with different formulation of the tirzepatide. Although, several methods have been developed previously for this drug as reported, but this is the -cost, fast and more accurate as the samples have been prepared by less costly chemicals and procedures, like protein precipitation technique also, use of nitrogen for drying has shown better results compared to earlier methods.

**Keywords:** RP-HPLC Methodology, Tirzepatide, Human Plasma, Development, Validation.

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*Address for Correspondence:
Vaishnavi N. Bitne, Department of Quality Assurance, PRES'S College of Pharmacy (For Women), Chincholi, Nashik.

## INTRODUCTION

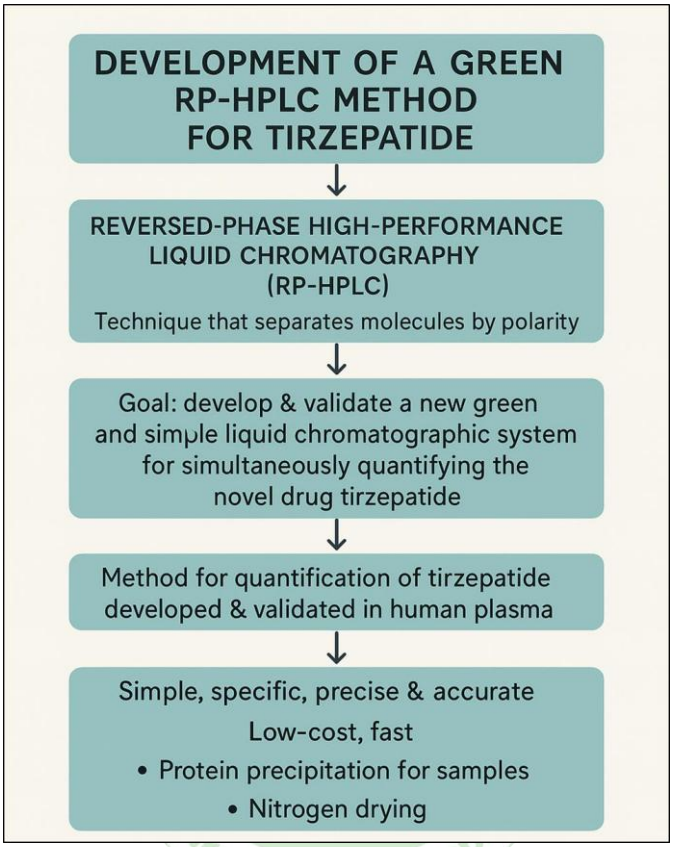
RP-HPLC for pharmaceutical analysis took off in the early 1970s with the introduction of commercially available microparticulate bonded packings. Although it rapidly became the dominant mode of chromatography in the pharmaceutical area, it quickly became apparent that the chromatography of basic compounds was not a straight forward matter. Despite this, RP-HPLC still figures prominently in both literature and pharmacopeia methods (1).

Reversed-phase HPLC (RP-HPLC) is the most commonly used mode of HPLC and, as the name implies, this mode is just the reverse of NP-HPLC, whereby the stationary phase is more nonpolar than the eluting solvent(2). Generally, RP-HPLC has a nonpolar stationary phase, e.g., C18 silica, and a moderately polar aqueous mobile phase. One common RP-HPLC stationary phase is surface-modified silica, $RMe_2SiCl$, where R is a straight chain alkyl group such as $C_{18}H_{37}$or $C_8H_{17}$. Silica-based reversed-phase sorbents are also known as "bonded-phase" materials (3).

The analysis of basic solutes using RP-HPLC methods presents a number of problems, principally because the analyte is retained by a number of retention mechanisms (some of which are poorly understood) in addition to the expected hydrophobic interaction (4). These include: hydrogen bonding, π–π interactions, ion exchange, ionpair formation and salting out. Itis the multiplicity of these retention mechanisms that often leads to poor chromatographic performance, characterized by low peak efficiencies, tailing (5).

Green Chemistry is the use of chemical techniques and methodologies that enable the elimination or reduction of the consumption, exposure or production of reagents, solvents, products and byproducts, and other compounds that are hazardous to the safety of humans or the environment. Hazards from chemicals classified as toxicity (acute and chronic), including carcinogenicity, mutagenicity, corrosivity, flammability, explosivity, as well as environment a impacts, such as atmospheric damage leading to global climate change Ideally, green chemistry means the avoidance of the application of hazardous and toxic solvents orreagents

during the conduction of analytical procedures as well as having a short analysis time with the consumption of safe, energy-efficient apparatus and devices The HPLC method was evaluated as a whole procedure and considered inexpensive due to the relatively low cost of the applied mobile phase, the relative availability of the HPLC-UV apparatus in many labs,especially the ones specialized in quality control(6). Also, the apparatus has a relatively low price in comparison with those which contribute to sensitive and high accuracy findings, including UPLC and LC-- MS-MS. It is important to high light that a short chromatographic run time is associated with the method, which supports the analysis of multiple samples routine steps–one factor at a time- for the subsequent optimization of the developed HPLC technique (7).




**Tirzepatide** is a dual GIP and GLP-1receptor agonist used for the treatment of type II diabetes in adults as an adjunct to diet and exercise. Tirzepatide is a novel dual glucose-dependent insulin tropic polypeptide (GIP) and glucagon-like peptide-1(GLP-1) receptor agonist (8). DualGIP/GLP-1agonists gained increasing attention as new therapeutic agents for glycemic and weight control as they demonstrated better glucose control and weight loss compared to selective GLP-1 receptor agonists in preclinical and clinical trials(9). Tirzepatide comprises a 39 amino acid linear synthetic peptide conjugated to a C20 fatty diacidmoiety. Its protein sequence was based on the sequence of endogenous GIP, and its pharmacological action on GLP-1 receptors is comparable to endogenous GIP.Glucagon-likepeptide-1(GLP-1) agonists are considered one of the most promising classes of diabetes drugs on the market(10). Tirzepatide, as next-generation GLP-1 drugs, not only stimulate insulin release in the body effectively controlling blood sugar levels, but also inhibit gastrointestinal motility and increase satiety. This technical note establishes a method for determining Tirzepatide drugs in plasma, providing a scientific basis for further research on drug safety and therapeutic efficacy(11).

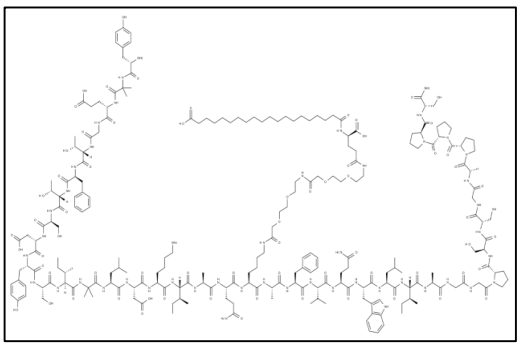


**Figure1:** Structureof Tirzepatide

## Medical uses:

Tirzepatide (as Mounjaro) is indicated to improve blood sugar control in adults with type 2 diabetes, as an addition to diet and exercise (12).

In a phase III trial, tirzepatide has demonstrated significant benefits in obese patients with a common type of heart failure: preserved ejection fraction (HFpEF).[24][25] Over two years, tirzepatide reduced the risk of major complications, including urgent heart failure visits, hospitalizations, increased diuretic treatment, and cardiovascular-related deaths, by 38% compared to placebo(13).

## Contraindications:

Tirzepatide is contraindicated for use in people with a personal or family history of medullary thyroid cancer and people with multiple endocrine neoplasia syndrome type 2(14).

## Adverse effects:

Preclinical, phase I, and phase II clinical trials indicated that tirzepatide exhibits adverse effects similar to those of other established GLP-1receptor agonists, such as dulaglutide (soldas Trulicity) and semaglutide (sold as Wegovy, Ozempic, and Rybelsus) (15).

These effects occur largely in the gastrointestinal tract.

The most frequently observed are nausea, diarrhea, and vomiting, which increased in incidence with the dosage amount (that is, the higher the dose, the higher the likelihood of side effects).

The number of patients who discontinued taking tirzepatide also increased as the dosage increased, with patients taking 15 mg having a 25% discontinuation rate vs 5.1% for 5 mg patients and 11.1% for dulaglutide.[clarification needed] To a slightly lesser extent, patients also reported reduced appetite(16).

Other side effects reported were dyspepsia, constipation, abdominal pain, dizziness, and hypoglycaemia(17).

## Pharmacology:

Tirzepatide is an analogue of gastric inhibitory polypeptide (GIP), a human hormone that stimulates the release of insulin from the pancreas (18).

Tirzepatide is a linear polypeptide of 39 amino acids that has been chemically modified by lipidation to improve its up take into cells and its stability to metabolism. It completed phase III trials globally in 2021(19).

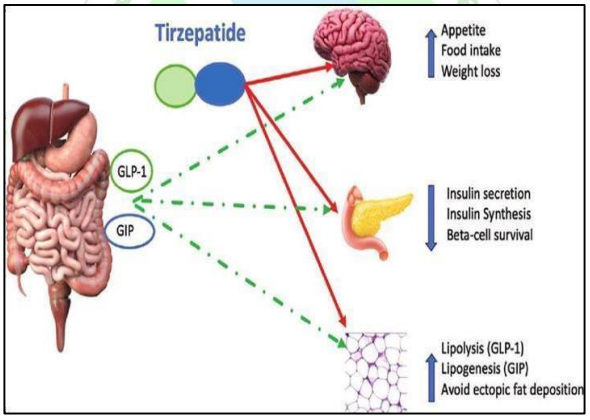



**Figure 2:** Mechanism of Action

## Materials and methods:

| | |
|---|---|
| Material | Material: Tirzepatide. |
| Method | Method:RP-HPLC |
| Chemicals | Tirzepatide Standard |
| Solvents | Water, acetonitrile, phosphate buffer solution (e.g.,0.1% Trifluoroacetic acid or phosphate buffe rat pH2-3) |
| Chromatographic Conditions | Human Plasma Using RP-HPLC: |
| Column | C18(4.6 mm x150mm,5 µm) |
| ColumnTemperature | 25-30°C |
| Mobile Phase: | Water(with 0.1% Formic Acid) or Phosphate Buffer (pH3-4):Acetonitrile or Methanol (with0.1% Formic Acid or similar additive) |
| Elution | Gradient Elution |
| Detector | UV Detector |
| Injection Volume | 10 µL to 20µL |
| Flow Rate | 1.0 mL/min |
| Run Time | 20-30min |

## Method validation:

Since, the HPLC method was developed, validation of the method by using various parameters was performed to ensure that the accomplishment of the method meets the requirements of the described bioanalytical applications. Following parameters were performed for method validation.

**Specificity:** Specificity is thecapability to evaluate the analyte distinctly in the presence of expected impurities and degraded products.

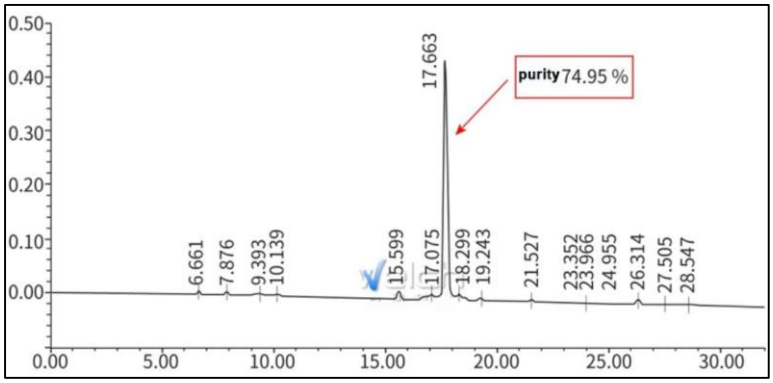



**Figure 3:** Chromatogram of Tirzepatide in human plasma

**Linearity:** From the experimental conditions described above, linear calibration curves of Metformin and levothyroxine were obtained for ten different concentrations level for both. There for metform in was 0.991 and for levothyroxine was 0.990. Linear correlations were found between peak area of Metformin and levothyroxine concentration and are described by the regression equation. The linearity range for Metformin and levothyroxine is 5-50 µg/ml.

Linearity of the developed method was evaluated by constructing calibration curves at six concentration levels over a concentration range of 50–1200ng/ml. The calibration curve was developed by plotting peak area versus concentrations (n = 6) using linear regression analysis with the help of the HPLC EZ chrome software. Good correlation coefficient 0.999 was observed. Other quantification parameters such as LOD and LOQ have also been calculated (Table1).To estimate the LOD and LOQ, blank methanol was applied six times and standard deviation of the analytical response was determined. The limits of detection (LOD) (signal/noise >3) and the limits of quantification (LOQ) (signal/noise >10) were determined by analyzing dilutions of a solution containing all the compounds. The sensitivity or LOD was calculated to be 33ng/ml while as lower limit of quantification was 40ng/ml and the mean retention times of Tirzepatide was 9.01 min. In order to reduce the matrix interference during the method validation, matrix match calibration experiment was performed over the concentration range of 50–1200 ng/ml.

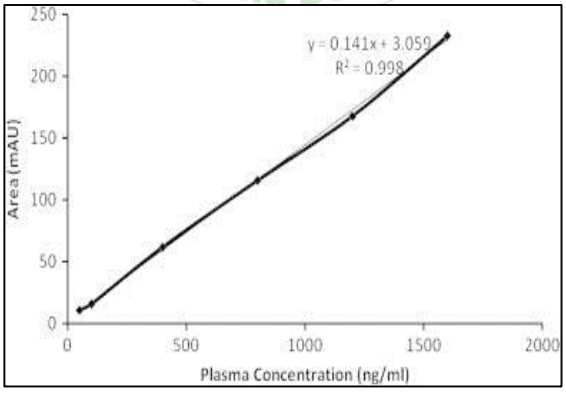



**Figure.4:** Standard curve of Tirzepatide in human plasma

## Precision and Accuracy:

The accuracy of an analytical method is the percentage of relativeness between the convention altruevalue and the value obtained by that method. Precision and Accuracy were determined by replicate analysis of known content of sample. The mean value should be within 15% of the actual value as per the acceptance criteria. The difference between mean amounts added and recovered (RE, %) serves as a measure of accuracy.

## Recovery:

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (5,25,50µg/ml).There coveries of metformin and levothyroxine were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

## Stability Studies:

The stability in human plasma over three freeze–thaw cycles and during short term, long-term, and post- preparative storage was tested by analysis of LQC and HQC samples. The freeze–thaw stability was determined over three freeze–thaw cycles within 3 days. Spiked plasma samples were frozen at -22$^{o}$C for 24h and thawed at room temperature in each freeze–thaw cycle. To study short-term stability, the frozen (- 22$^{o}$C) and then thawed plasma samples were kept at room temperature for 6 h before sample preparation. The results obtained from these test samples were compared with those from freshly thawed and processed samples (reference samples). Long-term stability was determined after keeping spiked plasma samples frozen at -22$^{o}$C for1 month. For this stability test the samples (test samples) were analyzed and the results were compared with those obtained from freshly prepared and processed samples (reference samples).The stability in stock solutions was studied after storage at 2$^{o}$C for 1 month. Three freeze thaw cycles of the quality control samples did not seem to affect quantification. Quality-control samples stored in a freezer at -22$^{o}$C were stable for at least 1month.Thawing of the frozen samples and keeping them at room temperature for 6 h had no effect on quantification. The stability in stock solutions was confirmed after storage for 29 days at 2$^{o}$C

## CONCLUSION:

A simple, specific, precise and accurate method for quantification of tirzepatide was developed and validated in human plasma and was applied for pharmacokinetics to determination the concentration of tirzepatide in human dosed with different formulation of the tirzepatide. Although, several methods have been developed previously for this drug as reported, but this is the low-cost, fast and more accurate as the samples have been prepared by less costly chemicals and procedures, like protein precipitation technique also, use of nitrogen for drying has shown better results compared to earlier methods. The stability studies conducted proved that the samples could retain their potency for up to 30 days, if stored under the stated conditions. Based on our results, the developed method features good quantification parameters and can serve as effective quality control method for standardization of drugs and pharmaceutical products.

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