Research Article

Bio-Analytical Method Development and Validation for Estimation of Tirzepatide by RP-HPLC in Dosage Form

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ABSTRACT

Background: Tirzepatide (TPZ) is a first-in-class dual glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist approved for the management of type 2 diabetes mellitus (T2DM). Accurate quantification of TPZ in biological matrices and dosage forms is essential for pharmacokinetic studies, therapeutic monitoring, and quality control. Objectives: The present study aimed to develop and validate a simple, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of Tirzepatide in human plasma and dosage form. Materials & Methods: Chromatographic separation was achieved using an Agilent C18 column (4.6 × 250 mm, 5 µm) with Acetonitrile:0.1% orthophosphoric acid (75:25 v/v) as the mobile phase at a flow rate of 0.8 mL/min. Detection was carried out at 252 nm with a 20 µL injection volume. The method was validated for linearity, accuracy, precision, robustness, LOD, and LOQ. The validated method was applied for the estimation of Tirzepatide in marketed formulation (Mounjaro injection). Results: The optimized chromatographic conditions yielded a sharp and symmetric peak at a retention time of 6.143 minutes. The method exhibited excellent linearity in the concentration range of 2.5–12.5 µg/mL with a correlation coefficient (R^2) of 0.999. Recovery studies confirmed accuracy with mean values between 99–101% and %RSD < 2. The LOD and LOQ were determined as 0.089 µg/mL and 0.271 µg/mL, respectively. Analysis of marketed formulation showed 99.19% of the labeled claim, within pharmacopeial limits. Conclusion: The developed RP-HPLC method was validated successfully and found suitable for the routine quantification of Tirzepatide in human plasma and pharmaceutical formulations.

KEY WORDS: Tirzepatide, RP-HPLC, Bioanalytical Method Validation, Human Plasma.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a progressive metabolic disorder characterized by chronic hyperglycemia resulting from insulin resistance and impaired insulin secretion.[1] With its rising global prevalence and associated complications, there is a continuous demand for innovative therapeutic agents and reliable analytical techniques to monitor their pharmacokinetics and ensure effective clinical outcomes.[2] Among the newer classes of antidiabetic agents, dual agonists targeting both glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptors have gained considerable clinical importance due to their unique mechanism of action and enhanced efficacy.[3,4]

Tirzepatide (TPZ) is the first-in-class dual GIP/GLP-1 receptor agonist approved for the management of T2DM. It not only improves glycemic control but also promotes weight reduction, thereby offering additional therapeutic benefits beyond glucose.[5,6,7] To support pharmacokinetic studies, therapeutic drug monitoring, and quality control of formulations, reliable and validated analytical methods for the quantification of TPZ in biological matrices are essential.[8,9] High-performance liquid chromatography (HPLC) remains one of the most widely applied techniques in bioanalytical research owing to its high sensitivity, accuracy, and reproducibility. Method validation, in line with International Council for Harmonisation (ICH) guidelines, is necessary to establish parameters such as linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ), ensuring that the developed procedure is robust and reliable for routine analysis.[10,11,12]

A literature review reveals that few analytical methods have been documented for the validation estimation of TPZ formulation. For estimation of TPZ a simple stability-indicating by HPLC and UPLC method for quantification of bulk drug and pharmaceutical formulations was developed.[13] Literature review also show that only limited analytical studies have been reported for TPZ, primarily using LC-MS/MS for quantification in biological samples.[14] However, to date, there is a lack of validated RP-HPLC methods for its estimation in human plasma and dosage forms.

The present study aims to develop and validate a simple, accurate, and precise RP-HPLC method for the estimation of TPZ in human plasma and dosage form. The method was optimized and validated according to ICH guidelines, and subsequently applied to analyze marketed formulations, demonstrating its applicability in routine laboratory and clinical research.

MATERIALS & METHODS

Chemicals and Reagents

The API sample of TPZ was obtained from Vtides Life Sciences Pvt Ltd. Injection of TPZ (Mounjaro injection 5 mg) were purchased from Eli Lilly and Company India Pvt Ltd. HPLC Grade methanol, Acetonitrile, Potassium Phosphate Buffer and Ortho phosphoric acid (OPA) were procured from Merck Ltd., India. Blood Plasma was obtained from the Red Cross, Jalgaon.

Instrumentation

The analysis of TPZ was performed using an Agilent Technologies HPLC Gradient System equipped with an auto-injector, diode-array detector (DAD), and UV730D absorbance detector, operated via Chemstation 10.1 software. A reverse-phase C18 column (Agilent, 4.6 mm × 250 mm, 5 µm) was used for chromatographic separation. Additional instruments employed during method development included a Thermo C18 column (150 mm × 4.6 mm, 5 µm), a UV-spectrophotometer (Analytical Technology), a VSI pH meter (Model VSI 1-B), a precision balance from A&D Company, Japan, and a sonicator from ENERTECH Electronic Instruments.

Chromatographic Conditions

Chromatographic separation was done by a C18 column using a binary gradient solvent system. Wavelength used for TPZ detection was 252 nm. Ambient temperature conditions were used throughout the experiment. The degassing of the mobile was done for 15 minutes. The 20 µL injection volume with a flow rate of 0.8 mL/min and solvent, Acetonitrile: 0.1 % OPA v/v (75:25%) was used after optimizing conditions.

Standard Stock Solution

An accurately weighed quantity, 5 mg of TZP was dissolved in Acetonitrile in a 5 ml volumetric flask and volume made up to 10 ml to produce a solution of 500 µg/ml.

Preparation of Blank Plasma

Blood samples are collected from human and then centrifuged at 5000 rpm for 1 hr to separate the plasma from blood. Then the separated was mixed with water then loaded on to the HPLC for Run.

Preparation of Calibration curve standard:

The above standard stock solution (500 µg/ml + 1 ml plasma) of TPZ was diluted with the mobile phase to yield five calibration curve standards with concentrations of 2.5, 5, 7.5, 10, and 12.5 µg/ml. The mobile phase was allowed to equilibrate with the stationary phase until a steady baseline was obtained. EGF samples were injected, and peaks were recorded at 252 nm.

Validation of Parameters

The validation of the developed method followed ICH guidelines for various parameters such as linearity, accuracy, precision, LOD, LOQ, and robustness.

Linearity

The linearity of the developed method was assessed by plotting a calibration curve by spiking plasma with a known quantity of TPZ ranging from 2.5-12.5 µg/mL. The regression equation was obtained from the calibration curve of peak area versus TPZ concentration. The correlation coefficient should not be less than 0.99.

Precision and Accuracy

The precision and accuracy of the developed method were assessed by spiking TPZ in human plasma. To assess intra-day precision, sample solutions of TPZ (2.5, 7.5, and 12.5 µg/mL) were examined on the same day at low, medium, and high concentration levels. The same concentrations were used to analyze inter-day precision. The method's accuracy was assessed through recovery studies conducted at three different concentrations (80%, 100%, and 120%). To perform the recovery studies, a specific concentration of the standard drug (80%, 100% and 120%) was added to previously analyzed sample solutions. %RSD and %recovery were calculated.

LOD and LOQ

The minimum detectable quantity in a sample, above background noise but not quantitated, is the LOD (Limit of Detection). The minimum quantity of substance in a sample that can be precisely and accurately quantified is the LOQ (Limit of Quantification). Three times the noise level was used to determine LOD, and ten times the noise level was used to calculate LOQ.

Robustness:

Robustness was examined by implementing slight modifications to the chromatographic conditions. The mobile phase composition was changed in (±1 ml/ min^{-1}) proportion and the proportion of Acetonitrile: 0.1 % OPA v/v (74:26 & 76:24 % v/v) in the mobile phase composition (±1ml/ min-1), and the change in detection wavelength (±1 ml/ min-1), and the effect of the results was examined.

Analysis of Marketed Formulation

To determine the content of TPZ in marketed formulation injection (Mounjaro injection 5 mg of TPZ), 5 ml plasma and 10 mL acetonitrile was taken. To ensure complete extraction it was sonicated for 15 min. 0.2 mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC, and drug peak area was noted. (Figure 4). Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of TPZ in the sample was calculated.

RESULT & DISCUSSION

Melting point: The procured reference standards of TZP were found to melt in the range of 221^0C.

λ max in UV spectrum of 10 µg/mL solution of TZP was found to be 252 nm.

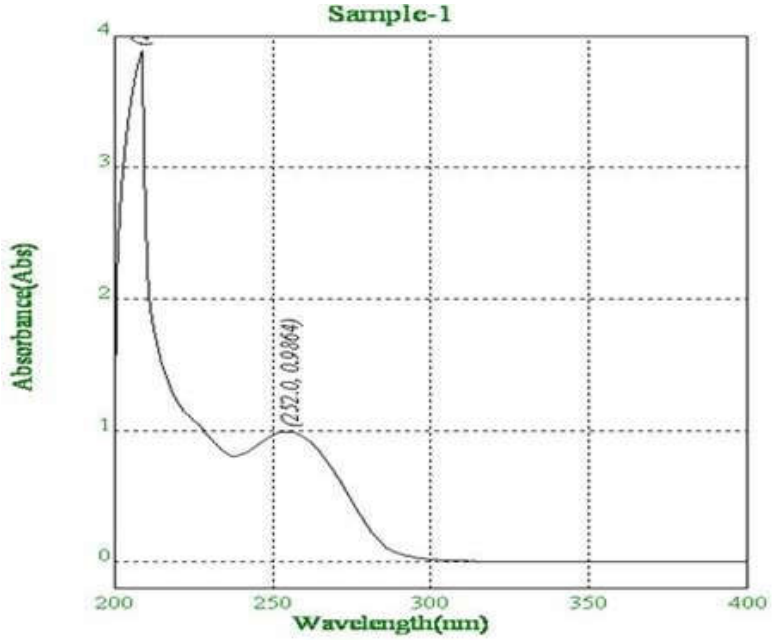

Figure 1: UV Spectrum of TPZ

Table 1: Chromatographic behavior of TPZ mobile phase of various compositions.

Run	Mobile Phase	RT	Remarks
1	Methanol: 0.1% OPA v/v (90:10 %), 252 nm, flow 0.7ml/min	3.147	No sharp peak
2	Methanol: 0.1 % OPA v/v (90:10%), PH3,252 nm, flow rate 0.7 ml/min	3.212	No Sharp peak
3	Acetonitrile: 0.01% OPA v/v (90:10%), PH3,252 nm, flow rate 0.8 ml/min	4.677	No Sharp peak
4	Acetonitrile: 0.1 % OPA v/v (80:20%), PH3, 252 nm, flow rate 0.8 ml/min	4.107	No sharp peak
5	Acetonitrile: 0.1 % OPA v/v (75:25%), PH3, 252 nm, flow rate 0.8 ml/min	6.143	Sharp Peak obtain

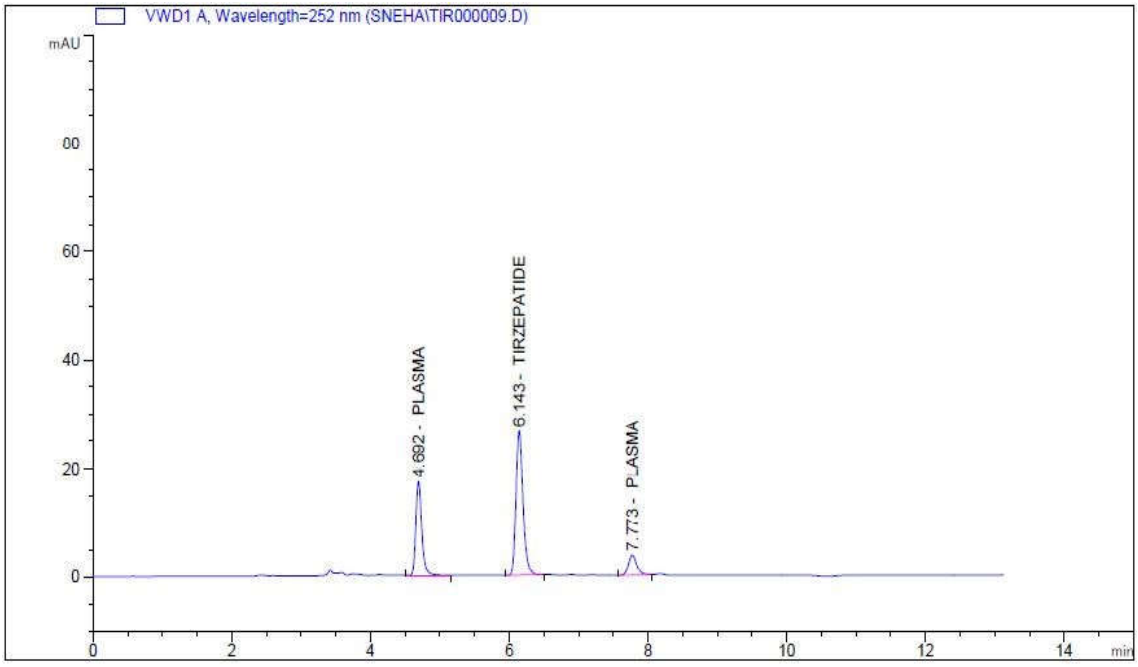

Figure 2: Trail 5 Chromatogram of TPZ in Acetonitrile: 0.1 % OPA v/v (75:25%), PH3, 252 nm, flow rate 0.8 ml/min chromatographic conditions

System Suitability Test

Based on system suitability parameters such as resolution, tailing factor, retention time and theoretical plates trial run 6 was selected to ensure the reproducibility of the chromatographic system. All the results were found to be within specifications proving the suitability of the method.

Table No 2: System Sutability Parameters

No.	RT [min]	Area[mV*s]	TP	TF	Resolution
1	6.215	74.81213	18235	0.74	-

Validation Parameters

Calibration Curve & Linearity

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 2.5-12.5 µg/mL for TPZ (**Table No 3)** depict the calibration data of TPZ. The respective linear equation for TPZ was y = 27.89X+5.944, where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of TPZ is depicted in (**Figure No 3).**

Table No 3:

Method	Conc (µg/ml)	Mean Area±SD (mV*sec)	% RSD of Peak Area
RP-HPLC Method	2.5	75.39±0.81	1.08
	5	143.41±0.65	0.45
	7.5	218.38±0.08	0.04
	10	285.53±0.13	0.04
	12.5	352.99±2.11	0.6

Calibration Curve

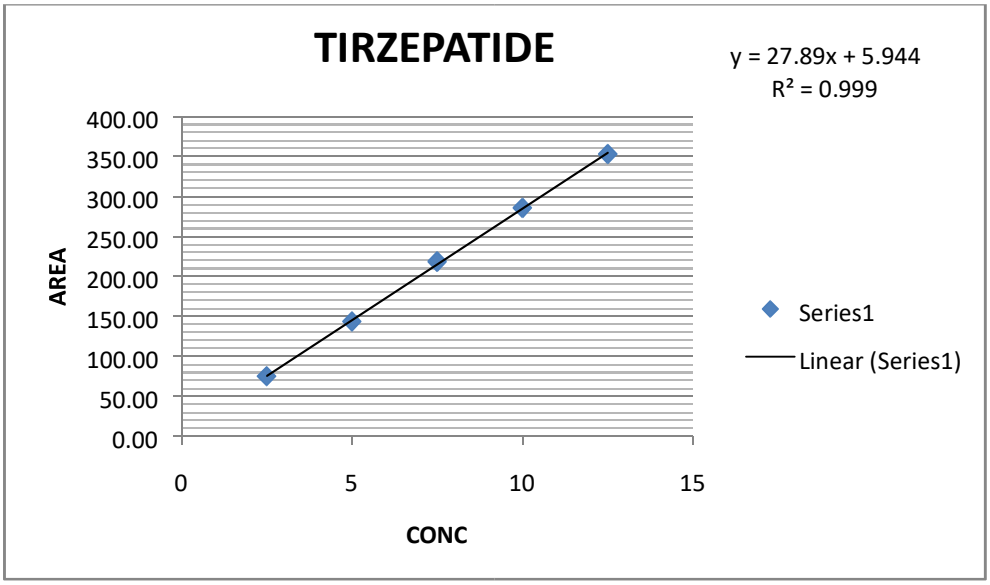

Figure No. 3: Calibration curve of TPZ

Accuracy

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed (Figure No 5)**.** Statistical validation of recovery studies shown in **(Table No 4).**

Table No 4: Results of Accuracy studies (*n*=3).

Level (%)	Amt. Taken (µg/mL)	Amt. Added (µg/mL)	%Recovery Mean ± SD	% RSD
80%	5	3	99.70±0.25	0.25
100%	5	5	101.34± 0.53	0.53
120%	5	12	101.18 ±0.42	0.42

Repeatability

Repeatability studies on RP-HPLC for TPZ was found to be 100.4 % (Table No 5). The %RSD was less than 2%, which shows a high percentage amount found between 98% to 102%.

Table No 5: Repeatability Results

Replicate (10 µg/ml)	Peak Area (mV*s)	Amount Found (µg/ml)
1	287.37	10.06
2	285.75	10.03
3	285.54	10.03
	Mean	**10.04**
	SD	**0.01**
	%RSD	**0.17**
	%Recovery	**100.40%**

Intraday and Interday Precision

The method was established by analyzing various replicates standards of TPZ using biological fluid. All the solutions were analyzed thrice to record any intra-day & inter-day variation in the result that concluded. The results obtained for intraday & interday are shown in **(Table No 6)** respectively.

Table No 6: Result of Intraday and Interday Precision Studies

Conc (µg/ml)	Intraday Precision			Interday Precision		
	Mean± SD	%Amt Found	%RSD	Mean± SD	%Amt Found	%RSD
2.5	75.08±0.23	99.15	0.31	75.29±0.11	99.45	0.14
7.5	218.51±2.53	101.62	1.16	218.43±0.16	101.58	0.07
12.5	354.12±4.45	99.87	1.26	351.26±0.34	99.05	0.1

Robustness

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done (Table No 7) and found in limit. The changes were did in mobile phase composition (±1 ml/ min-1), and wavelength (±1 ml/ min-1). %RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded

Table No 7: Robustness Results

Parameters		RT [min]	Area[mV*s]	TP	TF	Mean ±SD	%RSD
Acetonitrile:0.01% OPA v/v	74:26	6.447	74.9115	17033	0.74	75.05±0.21	0.28
	76:24	6.564	73.01477	16949	0.64	73.01±2.83	0.87
Wavelength change (nm)	251	6.819	79.59645	18535	0.82	79.7±0.78	1.01
	253	6.819	80.66449	18923	0.91	80.03±0.89	1.11

LOD and LOQ

The detection limit for TPZ was found to be 0.08952 µg/mL while the quantification limit was found 0.27128 µg/mL.

Analysis of Tablet Formulation

Analysis of marketed formulation of TPZ was found 99.19 %. Label claim was 99-101%.

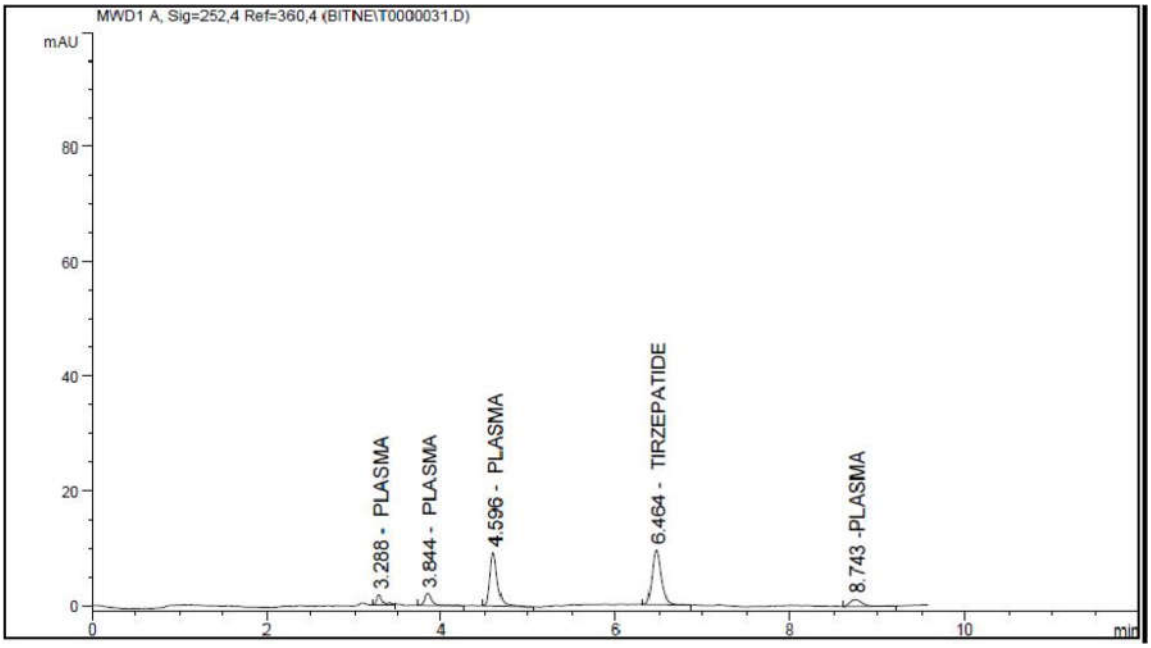

Figure No 4: Chromatogram for Marketed Formulation

Table No 8: Result of TPZ Marketed Formulation

RT [min]	Area[mV*s]	TP	TF	% Found	SD	%RSD
6.464	71.01292	19367	0.74	99.19	0.012	0.102

DISCUSSION

The present study successfully developed and validated a simple, precise, and robust RP-HPLC method for the quantification of TPZ in spiked human plasma and dosage form. The chromatographic conditions were optimized to achieve sharp peak symmetry and appropriate retention time, with Acetonitrile and 0.1% OPA (75:25 v/v) providing the best resolution (Table No 1 and 2, Figure No 2). The λmax of TPZ was found to be 252 nm (Figure No 1), consistent with previously reported UV absorbance characteristics of peptide-based drugs, confirming the suitability of this detection wavelength for routine analysis.

Linearity studies demonstrated excellent correlation ($R^2 = 0.999$) in the concentration range of 2.5–12.5 µg/mL, indicating the method's reliability for quantitative analysis across a wide therapeutic range(Table No 3). Recovery studies at 80%, 100%, and 120% levels yielded results within the acceptable range (99–101%), validating the accuracy of the method in spiked plasma

samples(Table No 4). Furthemore, the low %RSD values (<2%) observed in repetability and intra-day, inter-day precision confirmed that the method is highly reproducible (Table No 5 and 6).

The method's sensitivity was reflected by the low LOD (0.089 µg/mL) and LOQ (0.271 µg/mL), suggesting its potential applicability in pharmacokinetic and bioequivalence studies where detection of trace drug concentrations is essential. Robustness studies further confirmed that small, deliberate changes in chromatographic parameters such as wavelength and mobile phase composition did not significantly affect system performance, highlighting the method's stability and reliability under variable laboratory conditions(Table No 7).

Analysis of marketed formulation (Mounjaro® injection) showed drug content of 99.19%, which falls within the pharmacopeial limits of 98–102%, thereby confirming the applicability of the validated method for routine quality control(Table No 8, Figure No 4). Compared with previously reported LC-MS/MS methods for TPZ estimation, the present RP-HPLC method offers a cost-effective and accessible alternative without compromising sensitivity and reproducibility, making it suitable for laboratories with limited resources.

Overall, the developed RP-HPLC method fulfills the validation requirements set by ICH guidelines and demonstrates strong potential for application in pharmaceutical quality control, bioanalytical studies, and therapeutic drug monitoring of TPZ.

CONCLUSION

A simple, accurate, precise, and robust RP-HPLC method was successfully developed and validated for the estimation of TPZ in human plasma and dosage form. The method demonstrated excellent linearity, high recovery, reproducibility, and sensitivity, with %RSD values well within acceptable limits as per ICH guidelines. The low LOD and LOQ values highlight the applicability of the method for detecting and quantifying TPZ even at trace levels, making it suitable for pharmacokinetic and therapeutic monitoring studies.

Furthermore, the validated method was successfully applied to the analysis of a marketed formulation, confirming its reliability for routine quality control. Compared to more sophisticated techniques such as LC-MS/MS, this RP-HPLC method provides a cost-effective and accessible alternative without compromising analytical performance.

In summary, the developed method offers a reliable tool for routine laboratory use, bioanalytical applications, and pharmaceutical research involving TPZ**.**

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%: Percentage; ºC: Degree celcius; µg: Microgram; n: No. of determinations; mL: Milliliter; mg: Milligram; nm: Nanometer; T2DM: Type 2 Diabetes Mellitus; GIP:Glucose-dependent insulinotropic polypeptide; GLP-1:Glucagon-like peptide-1; min: Minute; RSD: Relative standard deviation; SD: Standard deviation; TPZ: Tirzepatide; hr: Hour; UV: Ultraviolet; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; ICH: International Council for Hormonization; LOD: Limit of detection; LOQ: Limit of quantification.

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