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Development and Validation of Simplified RP-HPLC Method for Quantification of Trelagliptin in Tablet Dosage Form: Greenness Analysis using AGREE Penalties

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Abstract: A simple and sensitive analytical method was developed to quantify the trelagliptin in tablet formulation. The analyte was separated on a Zorbax Eclipse XDB C_{18} column" (dimensions: 150 mm × 4.6 mm, 5 µm) using a mobile phase of HPLC grade water and methanol (40:60 % v/v) pumped at 1.0 ml/min. Detection of the effluent was done using a UV detector at a wavelength of 225 nm. The retention time for trelagliptin was 4.925 min. The drug showed linearity within the concentration range of 7.5–45 µg/ml. The accuracy of the method was considered satisfactory and the mean recovery percentage in the acceptable range of 99.4-100.2 %. The method was successfully validated according to ICH guidelines. The AGREE software was used to assess the environmental friendliness score of the proposed method, which was determined to be 0.8. The proposed method was simple, precise, sensitive, rapid, and robust for estimating trelagliptin in tablets.

Keywords: Trelagliptin, Type 2 diabetes mellitus, DPP-4 inhibitor, RP-HPLC, Validation, Formulation

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Introduction

Type 2 diabetes mellitus (T2DM) is a long-term disease characterized by the inability of the body to properly maintain blood glucose levels. This can take place because pancreatic beta cells do not produce enough insulin and body tissues develop insulin resistance. T2DM is cited to contribute about 90% of the total diagnosed diabetes. These are the causal factors; diet, obesity, genetic makeup, and sedentary lifestyles. In the last ten years, diabetes has been a leading cause of death

claiming the lives of millions of people, more so in LMICs. Diabetes is a chronic disease that has affected global population with an estimate of 425 million people living with the disease, and this statistic is expected to increase to 629 million people in the year 2045; expects an increase in global cases by 50% in next one decade (Kaku *et al.*, 2020). Diabetes involves numerous implications, nephropathy, neuropathy, retinopathy, and macrovascular diseases embracing stroke and/or heart disease (Kaku *et al.*, 2020). Also, diabetes has complications in the eye, leading to blindness; in the feet and legs, leading to amputations; and in the kidneys, resulting in kidney failure. T2DM is first treated by enforcing changes in the patient's behaviour like exercising more, doing away with certain types of food, and losing some weight. The handling of type 2 diabetes mellitus is intricate nonetheless; the major goal of the treatment is the achievement of good glycemic control and delay in the onset of issues related to the disease (Suh *et al.*, 2015). The aspect of blood sugar levels' management in patients with chronic kidney disease (CKD) has yet another complexity introduced to it. Diabetes is a global public health issue since it is identified as one of the principal causes of chronic kidney disease attributed to high sugar levels (Betônico *et al.*, 2016; Masakane *et al.*, 2018). Both diabetes and its comorbid CKD raise the mortality rates primarily because of their connection with cardiovascular diseases (Hirata *et al.*, 2017). Dipeptidyl peptidase-4 (DPP-4) inhibitors have a small risk associated with them and hypoglycemia because their mode of action depends on the concentration of glucose in the blood. These inhibitors are being used more frequently to manage patients with T2DM with moderate to severe nephropathy (Abe *et al.*, 2015).

DPP-4 is a serine protease that metabolizes the incretin hormones glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (Drucker *et al.*, 2006). It has a crucial function in maintaining glucose homeostasis. Noncompliance with medication is often low among patients with T2DM, and is a prevalent concern in clinical settings. A weekly dosage of a DPP-4 inhibitor has been deemed beneficial for treating patients with T2DM and CKD, as adherence to medication decreases with higher dosages and more prescription drugs (Kaku *et al.*, 2015). Trelagliptin (TGP), also known as SYR-472 or zafatek, is a newly developed member of DPP-4 inhibitor that has been authorized for use in Japan in 2015 (Grimshaw *et al.*, 2016). It is taken once a week. In a clinical trial, trelagliptin demonstrated effectiveness and a safe profile when administered once weekly to patients with T2DM. This is in contrast to other DPP-4 inhibitors now available on the market, which require daily or multiple daily doses. TGP is 2-[[6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxopyrimidin-1-yl]methyl]-4-fluorobenzonitrile ($C_{18}H_{20}FN_5O_2$), and its structure is shown in Figure 1.

In the past, numerous techniques have been established for the single or simultaneous measurement of TGP utilizing a variety of instruments, including LC/MS (Li Zhou *et al.*, 2020; Shereen *et al.*, 2021), UHPLC-MS/MS (Xiao-xia Hu *et al.*, 2016), HPLC (Qi Wang *et al.*, 2015; Anerao *et al.*, 2016; Luo *et al.*, 2018; Malleswar *et al.*, 2019), and UV spectrophotometers (Zaghary *et al.*, 2017; Shereen *et al.*, 2018). However, it was discovered that the current RP-HPLC method for TGP quantification in dosage forms was easy to use, accurate, quick, and affordable. Numerous studies in the literature reported on various RP-HPLC techniques for TGP estimation, but they were found to be laborious and time-consuming. But in order to promote green chemistry, the current work sought to establish a novel, sensitive, accurate, affordable, and stability-indicating RP-HPLC method for the determination of TGP in tablet dosage form (Zafatek® 100) using as few harmful chemicals as possible. In recent years, it has been advised that any HPLC technique development for active pharmaceutical substances have a good peak separation between the drug and its degradation products (Harshitha *et al.*, 2023; Sai *et al.*, 2023; Sumalatha *et al.*, 2024; Srinivas *et al.*, 2024). In compliance with ICH Q2 (R1), the developed RP-HPLC technique for TGP

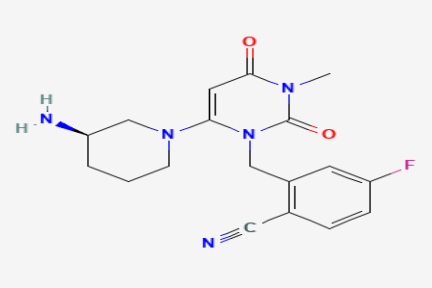

Fig. 1: Structure of Trelagliptin.

was validated. During the validation, the following parameters were assessed: limit of detection (LOD), limit of quantification (LOQ), linearity, specificity, accuracy, precision, and quantification of TGP in indicated tablet dosage form.

Materials and Methods

Chemicals and reagents:

Ascentyo Biosciences (Hyderabad, India) provided the TGP. Acetonitrile, methanol, and water were of the HPLC-grade solvents that were acquired from Merck Ltd. in India and used throughout the analysis.

Instruments:

The Shimadzu HPLC system (Model No. LC-20AD) working together with a UV detector (Model No. SPD-M20A) served as the basis for the study. Version 2 of the Empower program was used to acquire data. Included in the testing setup was a Zorbax Eclipse XDB C18 column, measuring 150 mm by 4.6 mm by 5 µm. A 20 µl sample loop-equipped Rheodyne injection valve was used to introduce the samples. An analytical balance made by Mettler Toledo was used for the weighing process.

Chromatographic conditions:

Using a mobile phase made up of water and methanol in a volumetric ratio of 40:60 v/v, an isocratic mode was employed. At room temperature (25°C), the analysis was carried out using a mobile phase flow rate of 1.0 ml/min. A 20 µl sample injection into the HPLC apparatus was necessary for each trial. UV detector was set up to identify TGP in the column's effluents at a wavelength of 225 nm (Fig. 2).

Mobile Phase preparation:

Following precise measurements, combined 400 ml of HPLC-grade water and 600 ml of methanol in a 40:60 v/v ratio. In an ultrasonicator, the mixture was allowed to degas for 10 min. After then, the mixture was vacuum-filtered through a 0.45 µ membrane filter.

Diluents preparation:

Acetonitrile and HPLC grade water were mixed well in a ratio of 50:50 (% v/v).

Preparation of standard stock solution:

Within a 100 ml volumetric flask (VF), 15 mg of TGP powder with a purity of over 99% was added. The diluent was then added to the flask, and it was sonicated for 20 min. The diluent was added, and the volume was adjusted to 100 ml. To get to a concentration of 30 µg/ml of TGP, 2 ml of the solution was then transferred to a 10 ml volumetric flask and filled up with 10 ml of diluent.

Preparation of sample solution from dosage form:

Twenty Zafatek® tablets (100 mg) were taken, and the tablets were crushed before being put inside a dry watch glass. Tablet powder equivalent to 15 mg of TGP was weighed and then added to a 100 ml volumetric flask along with 50 ml of diluents to create a standard stock solution of 200 µg/ml. The mixture was repeatedly sonicated for an hour to ensure the medication was fully soluble. Subsequently, a 0.45 µm membrane filter

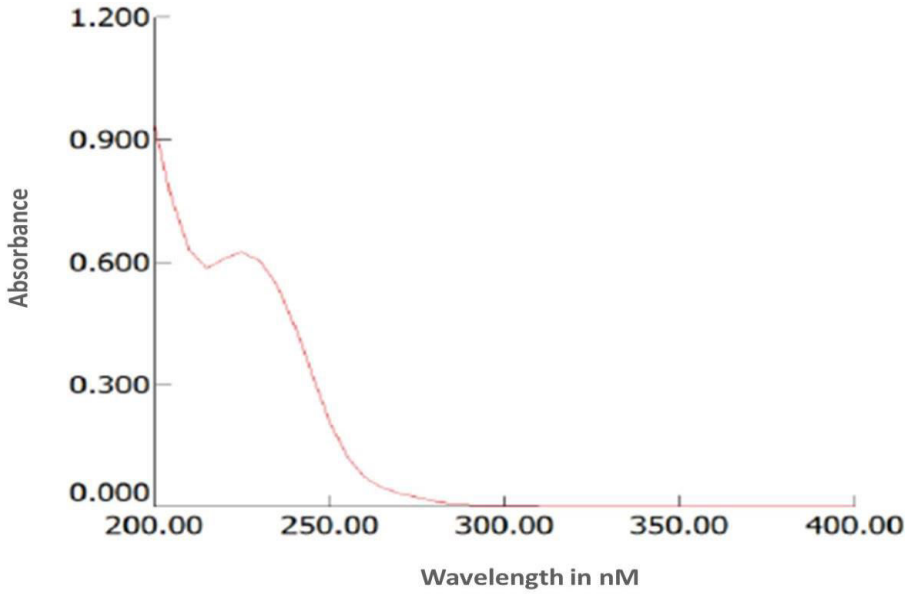

Fig. 2: UV spectrum of Trelagliptin.

was used to filter it, and 100 ml of diluent was added to make a stock solution. After that, 2 ml of the solution was added to a 10 ml volumetric flask along with 10 ml of diluent to reach a concentration of 30 µg/ml of TGP.

Selection of the detection wavelength:

For the HPLC analysis, a TGP solution of 30 µg/ml was employed. Using the mobile phase as a reference, the solution was examined using a UV spectrophotometer operating in the wavelength range of 190 to 400 nm. The goal of the scanning procedure was to find the wavelength at which the TGP most efficiently absorbs UV light in order to identify it for use in ensuing HPLC investigations. Figure 2 illustrates the drug's highest absorbance at 225 nm.

Analytical method development:

To create an HPLC analytical protocol for TGP, a number of parameters need to be optimized while keeping a set of constants in mind. To achieve the best chromatographic separation, thorough adjustments were made to the composition of the mobile phase, the choice of column, and the flow rates. However, to ensure consistency and streamline validation, a number of parameters were held constant, including the kind of detector, injection volume (20 µl), oven temperature (25 ± 2 ºC), and elution mode. For every set of chromatographic conditions, a spectrum at the chosen detection wavelength has been recorded. Additional criteria taken into account during the method's development included peak height, column pressure, accuracy, resolution, analysis time, and solvent efficiency per run.

Validation:

Following the ICH Q2 requirements (ICH), the method was verified once appropriate chromatographic conditions were established. Furthermore, the stability of solvents and reagents was also examined.

Evaluation of system suitability:

System suitability studies were carried out to confirm the HPLC system's reliability. To quantify column efficiency, plate count, and tailing factor, injections of 30 µg/ml were made six times. The outcomes showed consistency by verifying that the system achieved the predetermined standards and performed within the given parameters.

Specificity and selectivity:

Detecting TGP in the sample accurately and interference-free served as validation for the

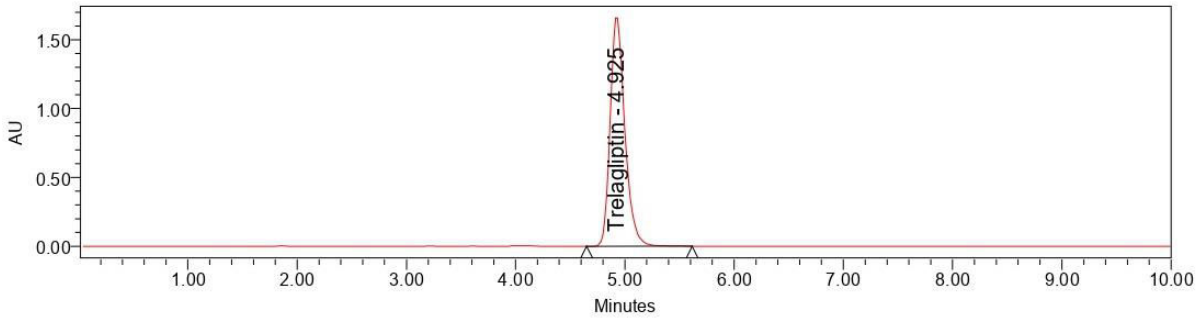

Fig. 3: Optimized chromatogram of Trelagliptin.

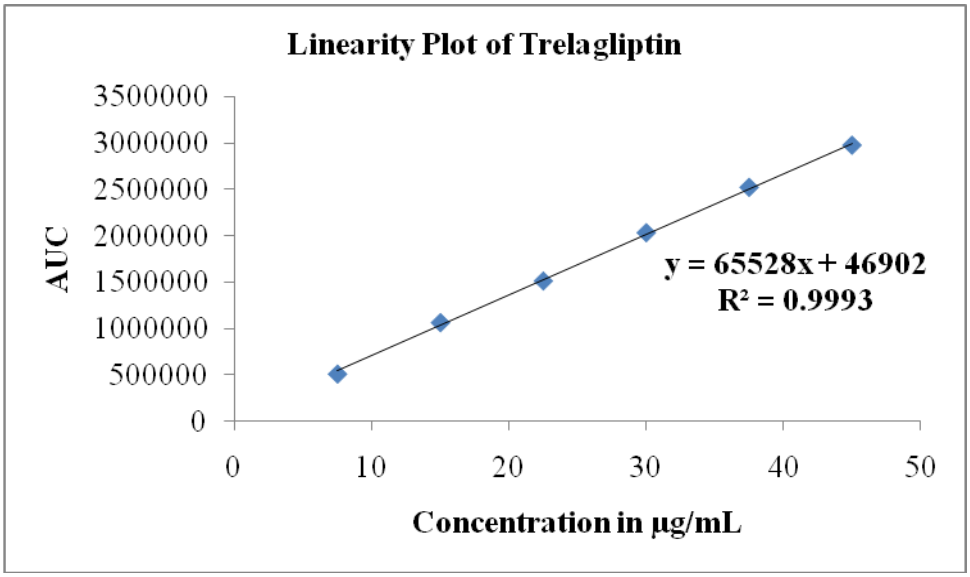

Fig. 4: Linearity graph of Trelagliptin.

specificity and selectivity of the approach. Only the diluent was used as the blank, and neither the TGP reference standard's chromatogram nor any interference were seen. Figure 3, correspondingly, shows the chromatogram for the standard.

Linearity:

The TGP's linearity was evaluated via generating dilutions from the standard stock solution that ranged from 7.5 to 45 µg/ml. During the HPLC process, peak area responses were measured for every concentration. Figure 4 depicts the linearity graph.

Precision:

Precision, or the consistency of results from several measurements, is an essential component of any analytical procedure. In compliance with ICH validation requirements, this study evaluated precision at multiple levels to guarantee a thorough assessment.

Intermediate precision (*IP*):

The capacity of a process to reliably produce accurate results in controlled environments in laboratories is referred to as intermediate precision. This covers variances in variables like different analysts, systems, days, and columns. Using the same batch of formulation, six samples have been generated in accordance with the test procedure and then injected. After analyzing the percentages of these samples, the percentage relative standard deviation (% RSD) of the outcomes was used to evaluate the method's robustness.

Accuracy or recovery studies:

A triple recovery investigation has been carried

out in order to confirm the HPLC procedure. Pre-analyzed samples were injected with 15, 30, and 45 µg/ml of TGP. To verify accuracy, the average recovery percentage was computed from these tests.

Robustness:

The robustness of the HPLC process was assessed by purposefully varying the wavelength and flow rate. As seen by negligible changes in the chromatogram, tailing factor, and plate count, the method's resilience was preserved despite fluctuations in flow rate and wavelength, retaining accuracy and precision.

Application of the developed method:

An efficient way to estimate the amount of TGP in commercially available tablets was to use the established HPLC method. The standard procedure described in the materials and methods section was followed in the production of the sample solutions. Three injections of each sample were made into the HPLC apparatus to ensure the accuracy and consistency of the results.

Solution and mobile phase stability:

The assay method's TGP solution stability was examined by keeping the sample and reference standard solutions in tightly-capped volumetric flasks at room temperature for 48 h. Every 12 h during the investigation, the same sample solutions were analyzed. The stability of the mobile phase was also assessed by comparing the newly made reference standard solutions to the freshly made sample solutions at 12-h intervals for up to 48 h. Throughout the duration of the investigation, the intended mobile phase remained unchanged. The RSD of the TGP test was calculated for the trials of solution stability and mobile phase. The stock solution was stored in a volumetric flask with a tight lid at 4°C for use in a subsequent study.

Greenness of the method:

AGREE® software version 0.5 beta, developed by the University of Vigo, Spain, was used to calculate the degree of greenness. It was accessible through an online repository (Pena-Pereira *et al.*, 2020; Madhavi *et al.*, 2024). Sample size, procedure stages, sample transformation level, derivatization, sample analysis rate per hour, organic phase ratio, number of hazardous compounds, energy consumption, and operator safety were among the seventeen different characteristics related to the strategy that were evaluated. The goal of the score and penalties is to assess any method in relation to geosafety and the human operating environment. Penalty points were visually depicted as a pictogram and were subtracted based on predetermined criteria. The final greenness score, which is obtained by deducting penalty points, is shown at the center of the spherical pictogram. A pictogram with a lot of green or a shade close to green indicates that environmental safety is high, while hues that are not green indicate that environmental safety is low.

Results and Discussion

Method development and optimization:

Mobile phase:

A trial-and-error approach was used to optimize the procedure and provide a chromatogram with the required resolution (R), efficiency, tailing factor, and number of USP plates. This method was repeated several times, with changes made to the composition of the mobile phase, the columns, and the flow rate. Using a Zorbax Eclipse XDB C_{18} column (dimensions: 150 mm × 4.6 mm, 5 µm) with a mobile phase made up of water and methanol (40:60 v/v) was the optimum process. Chromatographic separation was carried out using RP-HPLC with an isocratic elution mode at a flow rate of 1.0 ml/min. The process of trial and error produced ideal circumstances for TGP's well-separated peak (retention time: 4.925 min). Figure 3 displays the standard solution chromatogram.

Effect of column:

The elution of TGP was evaluated in a number of

columns, including C_4 and C_{18}. When the C_4 column was tested, a suitable peak form could not be obtained. Even at higher organic ratios in the mobile phase, TGP may exhibit a larger peak and tailing in the C_4 column due to its strong affinity for the stationary phase. The C_{18} column showed less retention time and an outstanding peak shape. The Zorbax Eclipse XDB column chemistry used in the C_{18} column showed better peak form and peak separation when compared to the Luna (Phenomenex) chemistry.

Effect of flow rate:

The TGP peak did not change noticeably when the flow rate was changed from 1 ml/min to 1.2 ml/min. As a result, the flow rate was kept at 1 ml/min.

Column oven temperature:

There was no apparent impact of the column oven's temperature on peak shape. An increase in temperature from 25° to 40°C barely affected peak morphology. The column oven temperature was kept at 25°C because a higher temperature could impair the column's lifespan.

System suitability parameters:

By estimating the percentage RSD (relative standard deviation) determined by six consecutive injections of the reference solution, the accuracy of the HPLC system was assessed. To be considered acceptable, the Relative Standard Deviation (RSD) may not be greater than 2%. The RSD of the standard solution was within the allowed range, indicating precision within the specified criterion, demonstrating the procedure's dependability for precisely quantifying TGP in samples. Table 1 presents the findings. TGP's retention time (RT) of 4.925 min demonstrated its quick recognition and successful separation. The TGP analysis HPLC method was tuned to select the right wavelength, fine-tune parameters, and mobile phase composition with care in order to accomplish quick analysis and good resolution. This extensive optimization satisfies stringent analytical criteria by ensuring precise and effective measurement of TGP.

Linearity:

For TGP, a linearity graph was generated with the area under the curve (AUC) on the y-axis and the concentration in µg/ml on the x-axis. Within the range of 7.5–45 µg/ml, a linear relationship between drug concentrations and peak area responses was discovered. The results highlight the importance of linearity in analytical methods (Table 2; Fig. 4). By examining peak areas, this function ensures precise assessment of drug levels across a wide concentration range. Precise TGP analysis is made possible by the HPLC approach's strong linearity in the defined concentration range and strong correlation between concentration and peak area response.

Precision:

The precision of the procedure was considered to be good, taking into account the repeatability of the sample and standard preparations. Table 3 provides a summary of the validation parameters. The outcomes show how dependable and consistent the HPLC method is when it comes to TGP analysis. The method may be reliably applied for accurate quantification in a variety of sample types.

Intermediate precision:

The adaptability of the HPLC method is demonstrated by its consistent performance across numerous labs, equipment, and analyzers—even on different days. Its moderate precision attests to its suitability for frequent usage, ensuring consistently accurate and reliable results under various experimental conditions. Table 4 demonstrates that the method and instrument's precision are demonstrated by the intermediate precision percentage RSD values, which are less than 2.0%.

Accuracy:

An average recovery percentage of 100.2% shows that the HPLC method is accurate in determining the TGP concentration within the expected range. The findings presented in Table 5, which display the mean recovery percentages and spiking

Table 1: System suitability parameters for Trelagliptin

S. No.	Parameter	Etifoxine	Acceptance criteria
1.	Retention time (RT)	4.925	--
2.	Theoretical plates (N)	8153	NLT 2000
3.	Tailing factor (T)	1.71	NMT 2.0
4.	Linearity range (µg/ml)	7.5-45	--
5.	Detection Limit (µg/ml)	0.05	--
6.	Quantification limit (µg/ml)	0.15	--
7.	Regression data: Slope	65528	--
8.	Regression data: Intercept	46902	--
9.	Regression data: Correlation coefficient	0.999	--

Table 2: Linearity of Trelagliptin

S. No.	Drug	Values of X and Y variables							Correlation coefficient
1.	ETX	Variable	1	2	3	4	5	6	0.999
		X	7.5	15	22.5	30	37.5	45	
		Y	509270	1061823	1510589	2030174	2518823	2971364	

Table 3: Precision study

S. No.	System Precision		Method Precision	
	Rt	AUC	Rt	AUC
1	4.946	2019728	4.962	2026300
2	4.943	2033599	4.946	2036507
3	4.946	2037312	4.947	2038196
4	4.953	2004096	4.952	2027967
5	4.934	2018253	4.95	2041296
6	4.951	2034994	4.939	2052987
Mean	4.9	2024663.7	4.9	2037208.8
SD	0.0	12923.0	0.0	9711.2
% RSD	**0.1**	**0.6**	**0.2**	**0.5**

concentrations validate the method's suitability for quantitative analysis by showcasing the effective recovery of TGP from various spiked samples. The accuracy of the recovery falling within the allowable range validates the method's satisfactory outcome in measuring the TGP content.

Robustness:

The results of extensive trials are shown in Table 6, which also describes the various variables that were looked at and how they impacted the method's effectiveness. The HPLC method's established reliability attests to its suitability for routine usage and guarantees consistent and reliable results even in the event of considerable modifications to the operating parameters. As seen by the small variations in peak areas and retention times, the method can yield reliable results in a variety of conditions. This HPLC

Table 4: Intermediate precision or Ruggedness study

Analyst Name	**Analyst I**			**Analyst II**		
Area of Std.	2037343			2070773		
S. No.	**Concentration (µg/ml)**	**AUC**	**Assay (%)**	**Concentration**	**AUC**	**Assay (%)**
1	30	1996927	98.0	30	2063797	99.7
2	30	2051449	100.7	30	2030149	98.0
3	30	2050980	100.7	30	2044490	98.7
4	30	2054331	100.8	30	2050983	99.0
5	30	2067833	101.5	30	2052641	99.1
6	30	2059675	101.1	30	2064693	99.7
	Mean	2046865	100.5	**Mean**	2051125	99.0
	SD	25263.6	1.2	**SD**	12891.9	0.6
	% RSD	1.2	1.2	**% RSD**	0.6	0.7
Difference between mean assay of two different analysts = 1.5 %						

Table 5: Accuracy study

S. No.	**Level**	**Amount added (µg/ml)**	**Mean Amount recovered (µg/ml)**	**Mean % Recovery**
1.	50%	15	14.91	99.4
2.	100%	30	30.08	100.2
3.	150%	45	45.11	100.2

Table 6: Robustness Study

Parameters	**Variation**	**Mean Peak area**	**%RSD**	**Tailing factor**	**No of Theoretical Plates**
Wavelength minus	220 nm	2095287	0.84	1.54	6223
Wavelength plus	230 nm	2099111.3	0.72	1.58	6427
Flow rate minus	0.8 min/ml	2064899.8	0.60	1.14	6115
Flow rate plus	1.2 min/ml	2079480.3	0.74	1.14	6910
Organic phase ratio change (less)	Water: Methanol (50:50)	2096724.8	0.38	1.55	6125
Organic phase ratio change (more)	Water: Methanol (30:70)	2086083.9	0.45	1.55	7089
Column change	Merck C_{18} column (250 mm × 4.6 mm × 5 µm)	2089486.1	0.25	1.54	7038
Temperature minus	20 °C	2103215.8	0.27	1.05	6185
Temperature plus	30 °C	2115257	0.61	1.20	6651

Table 7: Analysis of marketed formulation

Commercial Formulation	Ingredients	Labeled Amount (mg)	Amount Found (mg)	Found %
Zafatek®	Trelagliptin	100 mg	99.47 mg	99.47

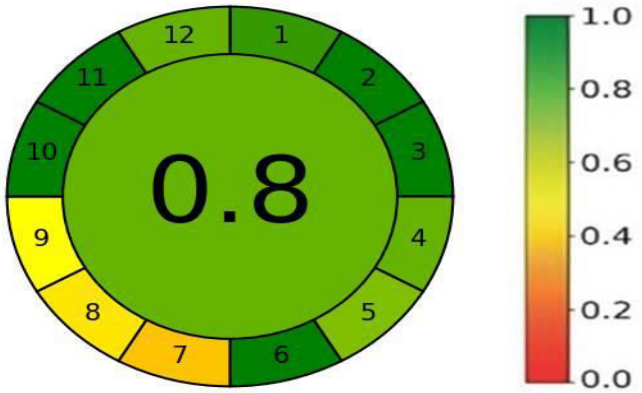

Fig. 5: Greenness of the developed method using AGREE software.

technique differs from previous analytical procedures in that it has shorter retention times, more theoretical plates (indicating enhanced resolution), and a mobile phase that helps separate TGP more effectively from other components. As such, its improved accuracy and efficiency make it more appropriate for routine TGP assessment in a variety of sample types.

Analysis of marketed formulation:

The TGP tablets' percentage assay, which reported out at 100 ± 15%, indicates that the tablets under examination have a percentage purity that is within the permitted range in accordance with ICH guidelines. Table 7 displays the results.

Solution and mobile phase stability:

The RSD of the TGP test was within 2% in both the mobile phase stability (0.4) and solution stability (0.1) trials. The findings of the tests into the stability of the assay sample solutions and mobile phase showed that they were stable for up to 48 h at room temperature and three months at 4ºC.

Greenness of RP-HPLC method:

The AGREE score of the created approach was determined to be 0.8, with a deduction of 0.2 penalty points (Fig. 5). This is due to the mobile phase's 60% v/v methanol content, which is flammable and known to have mild toxicity to aquatic life. Figure 3 illustrates that TGP has a retention period of less than 5 min in a single cycle, minimizing mobile phase waste. The extended sample analysis time in a single run in the literature resulted in a substantial loss of TGP. The lengthy sample analysis time in a single run contributed to a large increase in TGP waste in the literature. The created approach is deemed to be a green method based on its AGREE score.

Conclusion

The ICH requirements were followed in the development and confirmation of an RP-HPLC technique. The procedure involved the use of a UV detector (Model No. SPD-M20A) and a Shimadzu HPLC system, with 225 nm being the wavelength at which detection was accomplished. A Zorbax

Eclipse XDB C_{18} column (150 mm × 4.6 mm, 5 µm) with isocratic elution was used, with an injection volume of 20 µl. The final method is simple, accurate, fast, dependable, affordable, and sensitive. Just two of its numerous qualities are its short duration (less than 6 min) and excellent resolution. All validation parameters' % RSD values satisfied the necessary requirements, proving the method's suitability for regular trelagliptin laboratory analysis and quality control.

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