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RESEARCH ARTICLE
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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE STUDIES OF SODIUM-GLUCOSE CO-TRANSPORTER 2 (SGLT2) AND DIPEPTIDYL PEPTIDASE 4 (DPP-4) INHIBITORS EMPAGLIFLOZIN AND LINAGLIPTIN IN PHARMACEUTICAL DOSAGE FORM.

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Abstract:
A fixed-dose amalgamation pill having linagliptin and empagliflozin, which includes a sodium-glucose co-transporter 2 (SGLT2) inhibitor as well as a dipeptidyl peptidase 4 (DPP-4) inhibitor correspondingly, has gained approval recently. This study presents a developed RP-HPLC technique for measuring Empagliflozin and Linagliptin in pharmaceutical preparations. The analytical method has validated for key characteristics, including linearity, precision, robustness, and accuracy, in accordance with the rules set by the ICH. The RP-HPLC analysis performed using an ODS column with measurements of 250 mm in length and 4.6 mm in diameter, and a 5 µm particle size. The mobile phase consisted of an ammonium acetate buffer (0.770 g per 1000 mL) as well as acetonitrile in a 65:35 (v/v) ratio, with a flow rate set at 1.00 mL/min. The detection has been carried out using a PDA detector. The optimal detection wavelengths were determined to be 210 nm for Empagliflozin and 290 nm for Linagliptin. For Empagliflozin, the calibration curve demonstrated tremendous linearity within the range of conc. for 20-120 µg/mL, having outstanding regression coefficient (R^2 = 0.9999). Similarly, Linagliptin exhibited linearity over a conc. range of 4.0 to 24 µg/mL, also having a regression coefficient of 0.9999. Limits of detection (LODs) for Linagliptin as well as Empagliflozin has proven at 0.313 µg/mL and 1.48 µg/mL, respectively. LOQs were discovered to be 4.48 µg/mL for Empagliflozin and 0.949 µg/mL for Linagliptin. Accuracy of Empagliflozin ranged from 98.60% to 100.12%, Linagliptin's accuracy ranged from 99.62% to 99.90%. It was discovered that every system suitability parameter fell between reasonable bounds.
This method is efficient, rapid, as well as suitable for use in (QCL) quality control laboratories to analyze Linagliptin and empagliflozin in solid dosage forms for pharmaceuticals.

Keywords: World health organization, Limit of Detection, Limit of Quantification, International Commission for Harmonization, High Performance Liquid Chromatography.

Introduction

Diabetes mellitus is a potentially fatal condition that needs constant medical care.

T2DM (Type 2 diabetes mellitus), the progressive condition characterized by insulin resistance and deficiency of insulin [1]. Chronic hyperglycemia linked to diabetes can lead to serious complications such as kidney failure, amputations, blindness, and death.

Empagliflozin is utilized to enhance adult glycemic control individuals diagnosed by functioning as a sodium glucose inhibitor cotransporter-2 (SGLT-2) in people with type 2 diabetes. These co-transporters play a role in reabsorbing glomerular filtrate's glucose in kidneys. By inhibiting SGLT-2, empagliflozin decreases the renal absorption of glucose, thereby lowering the renal threshold for glucose and increasing glucose excretion. This mechanism helps in reducing hyperglycemia and blood pressure [2].

In August 2014, Empagliflozin was the latest addition to its class to receive approval from the Food and Drug Administration (FDA). Known for its relatively low side-effect profile, Empagliflozin is particularly effective when used alongside other antidiabetic medications [3]. Because the mechanism of action of empagliflozin is independent of both the insulin pathway and beta-cell function, there is very little chance of hypoglycemia [4].

It is recommended to assist individuals with type 2 diabetes in improving their blood sugar regulation. It works best if taken in conjunction with a healthy nourishment as well as frequent workout. The kidney is crucial in maintaining glucose balance by producing, utilizing, and, most significantly, reabsorbing glucose from the glomerular filtrate through SGLT2. SGLT2 is responsible for approximately 90% of renal glucose uptake. By inhibiting SGLT2, empagliflozin raises the excretion of glucose in urine (UGE), leading to lower plasma glucose levels without relying on insulin [5].

(2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl] is the IUPAC name for empagliflozin.-6-(hydroxymethyl)oxane-3,4,5-triol, having the molecular formula $C_{23}H_{27}CO_7$ and a molecular mass of 450.9 g/mol.

As depicted in Figure 1, the anti-diabetic medication empagliflozin works by preventing the sodium-glucose co-transporter 2 (SGLT2) from being active. Thereby exhibiting anti-hyperglycemic properties [6-9]. After being taken orally, Empagliflozin effectively and selectively inhibits SGLT2 in the kidneys, particularly in the proximal tubule. This action restricts the reabsorption of glucose. By blocking SGLT2, Empagliflozin (Figure: 1) promotes the excretion of glucose through urine by the kidneys. Consequently, this leads to a decrease in plasma glucose levels, without relying on insulin.

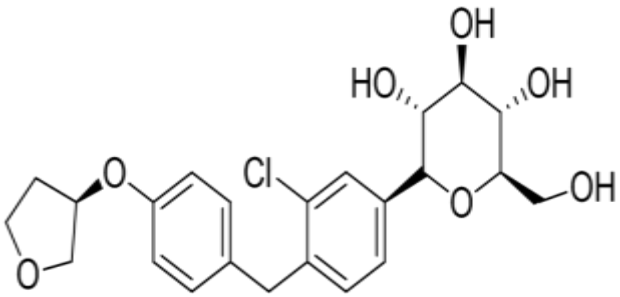

Figure 1: Chemical structure of Empagliflozin

Linagliptin (Figure: 2) is a compound with a xanthine base, characterized by molecular formula of $C_{25}H_{26}N_8O_2$ (mol. Weight: 472.54 g/mol). It features substituents at positions of 1, 3, 7, and 8, including (4-methylquinazolin-2-yl) methyl, methyl, but-2-yn-1-yl, and 3-aminopiperidin-1-yl, respectively. This medication is recognized as a potent, selective, and long-lasting inhibitor of DPP-4 [10, 11]. Its effectiveness can be attributed in part of their unique scaffold structure of xanthine [12].

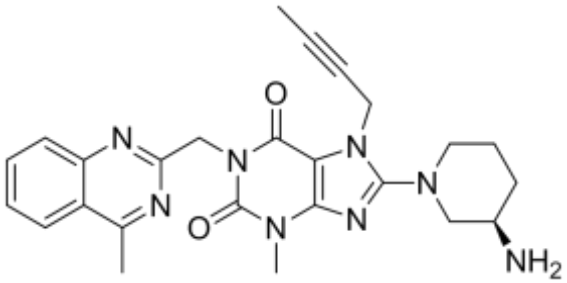

Figure 2: Linagliptin structure.

After conducting a literature review, it was discovered that different techniques have been employed to analyze Empagliflozin and Linagliptin in pharmaceutical drugs, including spectrophotometric methods, high-performance liquid chromatography (HPLC) and LC–MS/MS determination. The approach, outlined here, offers an efficient and rapid method that can applied in QCL to assess Linagliptin and Empagliflozin in solid dose for pharmaceuticals.

Experimental:

Instrument

The LC-2030C 3D series, a comprehensive high-performance Shimadzu liquid chromatography model.

This model makes use of a column oven, an autosampler, a PDA detector, and a pump with quaternary low-pressure gradient capability. Separations has conducted at an Inertsil ODS column measuring 250 mm x 4.6 mm with a particle size of 5 microns. We used Lab Solution software to record the chromatographic data, and we also used an ATX 224 Shimadzu analytical balance and an Elma Sonics Model E60H sonicator for degassing.

Chemicals and reagents

Analytical-grade chemicals was utilized in this study. Acetonitrile and ammonium acetate of HPLC gradient grade were supplied by Merck, Darmstadt, Germany. The Linagliptin and Empagliflozin drug formulations were given by the Ferozsons Laboratories in Pakistan's Nowshera.

Preparation of Buffer Solution:

A precisely weighed 0.770 g of ammonium acetate was poured into a 1000 mL volumetric flask. Ammonium acetate was dissolved in distilled water, and then diluted using the same solvent to the appropriate amount.

Mobile phase preparation:

The buffer was combined with acetonitrile to create the mobile phase in a 650:350 (v/v) ratio. This mixture was thoroughly combined, filtered through a degassed and used a 0.45-micron membrane filter using a sonicator.

Diluent Preparation:

Acetonitrile and distilled water were combined in a 700:300 (v/v) ratio to create the diluent.

Empagliflozin Standard Stock Solution Preparation:

30 mL of diluent and 50 mg of the working standard empagliflozin is added in a 50mL volumetric flask and sonicated until dissolved. The solution was then diluted to the mark with diluent, resulting in an amount of 1000.0 µg/mL.

Linagliptin Standard Stock Solution Preparation:
70 mL of diluent and 20mg of the Linagliptin working standard were combined in 100mL volumetric flask, and sonicated until dissolved. Subsequently, the solution was diluted using diluent until its concentration reached a 200.0 µg/mL.

Standard Solution Preparation:
Five milliliters each of the stock solutions for linagliptin and empagliflozin should be precisely transferred into a fifty milliliter volumetric flask. After adding diluent to the appropriate amount, filter the mixture using a 0.45-micron membrane filter.
This results in final concentrations of 100 µg/mL for Empagliflozin and 20 µg/mL for Linagliptin.

Sample solution Preparation:
Weigh all the twenty tablets separately, then determine the typical tablet's mass. Pulverise tablets to a fine powder, then weigh out one tablet's worth of Empagliflozin (25 mg) and Linagliptin (5 mg) and transfer the contents into a 100mL volumetric flask. Following a 15-minute shake and 3-minute sonication, add 70 mL of diluent. Use diluent to dilute the solution to 100 milliliters, then filter it through a 0.45 µm membrane filter, throwing away the first few milliliters. Transferring the filtered solution in 10 mL to a 25 mL volumetric flask, dilute to the appropriate level to obtain a concentration of 20 µg/mL Linagliptin and 100 µg/mL Empagliflozin.

Validation parameters for analytical methods:
ICH guidelines were utilized to verify the procedure and tests for accuracy, robustness precision, and linearity were conducted.

Conditions of Chromatograph
Separation was carried out using a standard ODS column (4.6 mm × 250 mm; 5 µm packing). Before being utilized, the mobile phase was filtered and degassed. It was made up of a 650:350 (v/v) ratio of acetonitrile and ammonium acetate buffer. The diluent is composed of water and acetonitrile at a ratio of 700:300 (v/v).
A steady 30°C was maintained for the column temperature. The chromatographic conditions were optimized using an injection volume of 10 µL. 12.0 minutes was the total run time, and the flow rate was set at 1.0 mL/min. A PDA detector was used for the detection of Empagliflozin at 210nm and Linagliptin at 290 nm.

Chromatogram:
Figure 3 depicts the chromatogram of diluent.

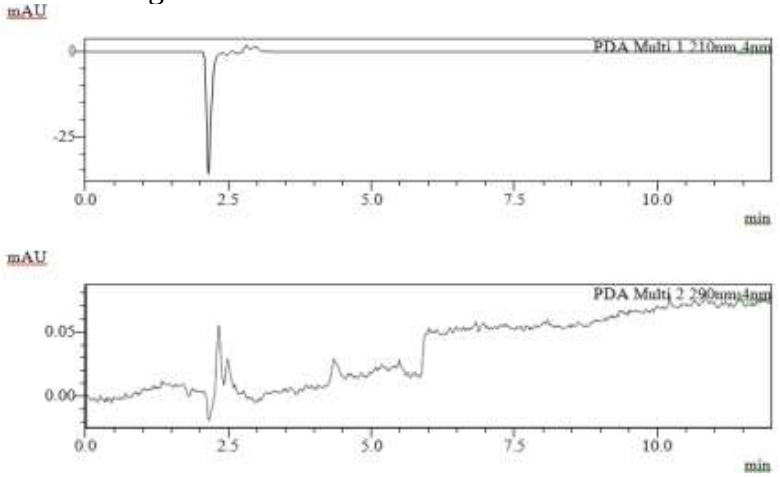

Figure 3: Empagliflozin and linagliptin's diluent (mobile phase) HPLC chromatogram

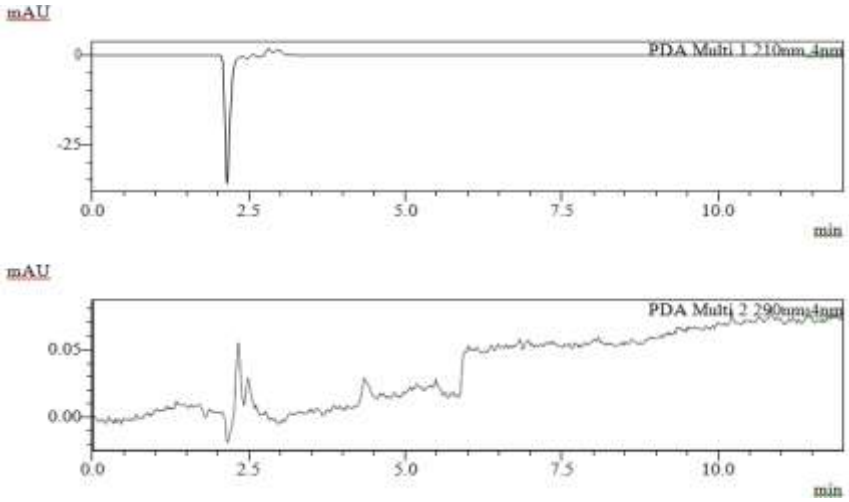

Figure 4: Placebo (Powder) HPLC chromatogram used for Linagliptin and Empagliflozin.

Figure 5 presents the standard chromatogram of 4 µg/mL and 20 µg/mL of linagliptin and empagliflozin, respectively.

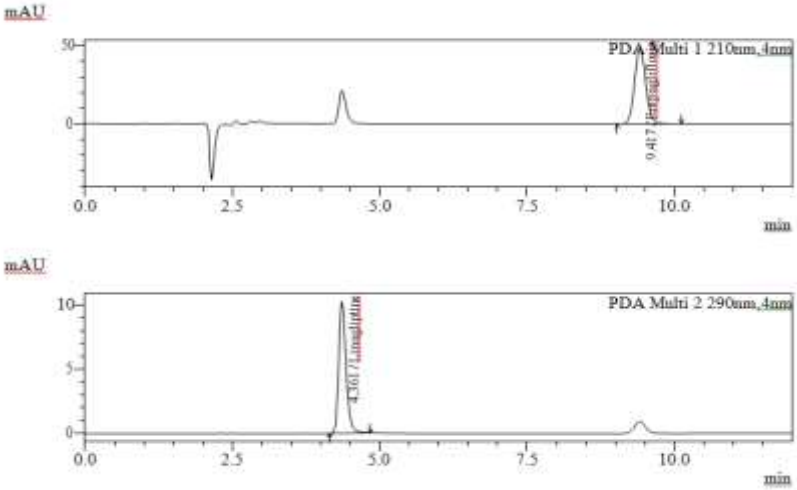

Figure 5: HPLC chromatogram of Linagliptin (4µg/mL) and Standard Empagliflozin (20µg/mL)

Figure 6 shows the chromatogram of standard Linagliptin and Empagliflozin at concentrations of 40 µg/mL and 8 µg/mL, respectively.

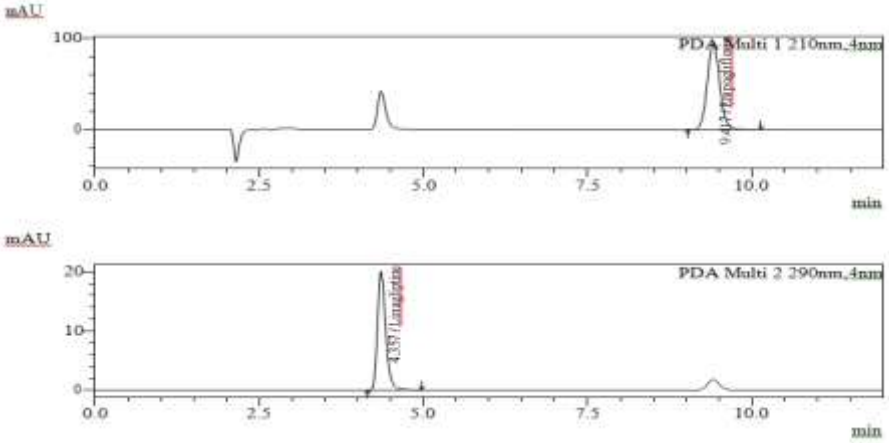

Figure 6: HPLC chromatogram of Linagliptin (8µg/mL) and Standard Empagliflozin (40µg/mL)

Figure 7 displays standard chromatogram of linagliptin at 12 µg/mL and Empagliflozin at 60 µg/mL, respectively.

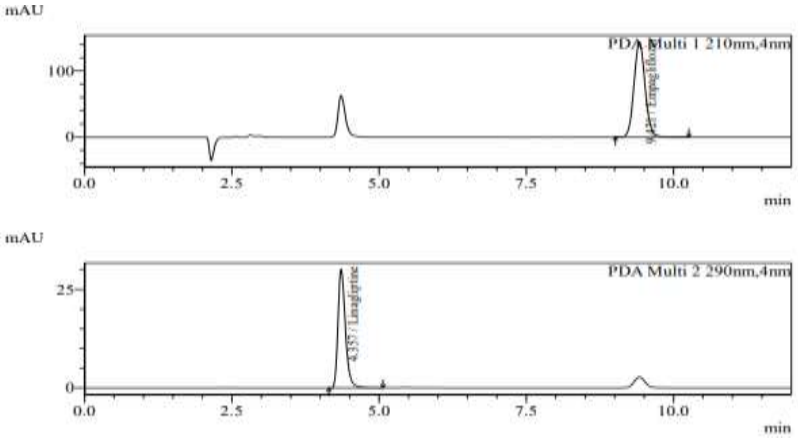

Figure 7: Linagliptin (12µg/mL) and Standard Empagliflozin (60µg/mL) HPLC chromatograms.

Figure 8 presents the standard chromatograms of 16 µg/mL and 80 µg/mL of linagliptin and empagliflozin, respectively.

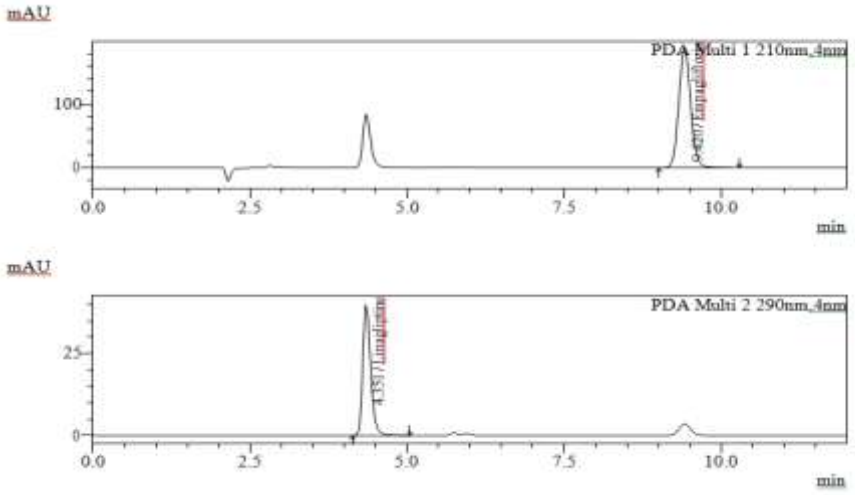

Figure 8: Linagliptin (16µg/mL) and Standard Empagliflozin (80µg/mL) HPLC chromatograms

Figure 9 shows the standard chromatograms of 20 µg/mL and 100 µg/mL of linagliptin and empagliflozin, respectively.

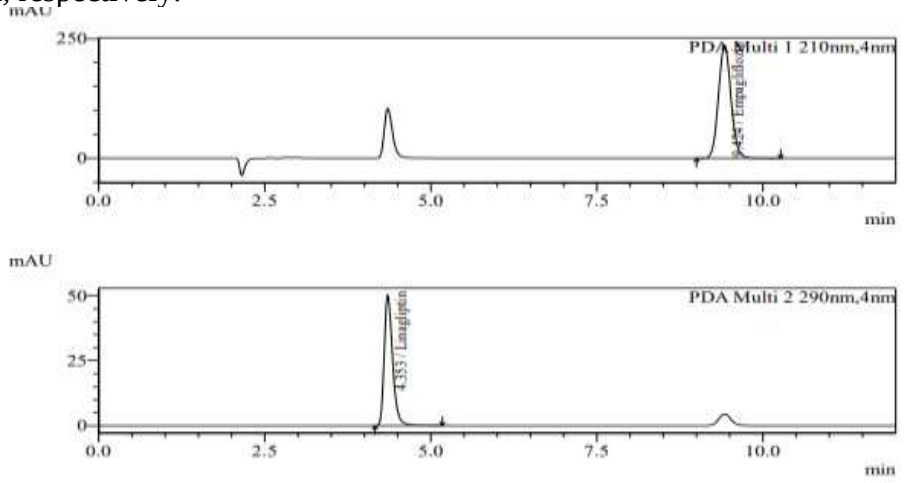

Figure 9: Linagliptin (20µg/mL) and Standard Empagliflozin (100µg/mL) HPLC chromatograms

Figure 10 displays the standard chromatograms for linagliptin and empagliflozin at 120 µg/mL and 24 µg/mL, respectively.

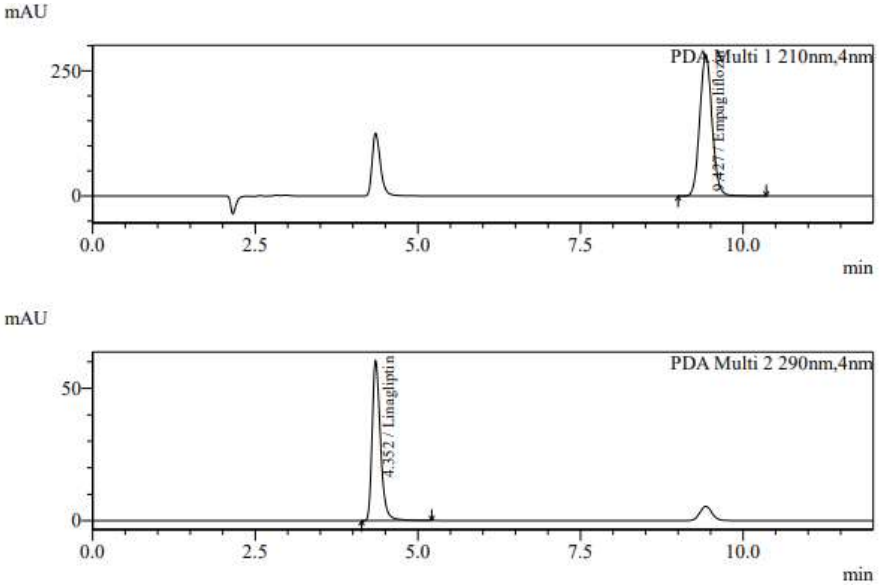

Figure 10: HPLC chromatogram for standard doses of 24µg/mL of linagliptin and 120µg/mL of empagliflozin.

Linearity:

At least six distinct dosages of both medications were used to test the linearity of the results. Table 1 shows that peak height grew in a linear fashion from 20% (the lowest conc.) to 120% (the highest conc.) of the expected working range. The linearity graphs are shown in Figure 11.

Dilution Factor Empagliflozin	Conc. of Solution (µg/mL) Empagliflozin	Area / Abs Empagliflozin	Dilution Factor Linagliptin	Conc. of Solution (µg/mL) Linagliptin	Area / Abs Linagliptin
2.40	120	3711273	1.2	24.0	537219
2.00	100	3105638	1.00	20.0	442767
1.6	80	2462737	0.8	16.0	354522
1.2	60	1884820	0.6	12.0	265059
0.8	40	1250049	0.4	8.00	176157
0.4	20	640970	0.2	4.00	88933
0.00	0.00	0.00	0.00	0.00	0.00
R^2		0.9999	R^2		0.9999
Y-intercept		14086.39	Y-intercept		-1230.07

Table 1: linear range for the HPLC analysis of particular medications.

Accuracy (%age Recovery)

Accuracy of the technique was assessed by incorporating a recognized quantity of Empagliflozin and Linagliptin to placebo powder. The protocol was followed for the creation of the sample and standard

solutions, and the percentage recoveries were computed using the given formula. The results are displayed in Tables 2 and 3, with graphical representations provided in Figure 12.

% Recovery = (µg/mL found)/ (µg/mL added) x 100

Nominal Content (%) Empagliflozin	Amount Added (µg/mL)	Area	Amount Found (µg / mL)	% Recovery	Mean Recovery
80	80.0	2470184	79.18878257	99.0	98.9
	80.0	2464347	79.00166092	98.8	
	80.0	2469565	79.16893877	99.0	
100	100	3109467	99.68281965	99.7	99.9
	100	3128195	100.283199	100.3	
	100	3115474	99.87539115	99.9	
120	120	3695140	118.4582355	98.7	98.6
	120	3677795	117.9021921	98.3	
	120	3696304	118.4955509	98.7	

Table 2: % Recovery for HPLC determination of Empagliflozin.

Nominal Content (%) Linagliptin	Amount Added (µg/mL)	Area	Amount Found (µg/mL)	% Recovery	Mean Recovery
80	16.0	357230	16.02797931	100.2	99.9
	16.0	355723	15.96036414	99.8	
	16.0	356084	15.97656127	99.9	
100	20	444274	19.93341679	99.7	99.9
	20	447341	20.07102509	100.4	
	20	444917	19.96226652	99.8	
120	24	534840	23.99687723	100.0	99.7
	24	530660	23.80933152	99.2	
	24	534797	23.99494793	100.0	

Table 3: % Recovery for Linagliptin determination using HPLC.

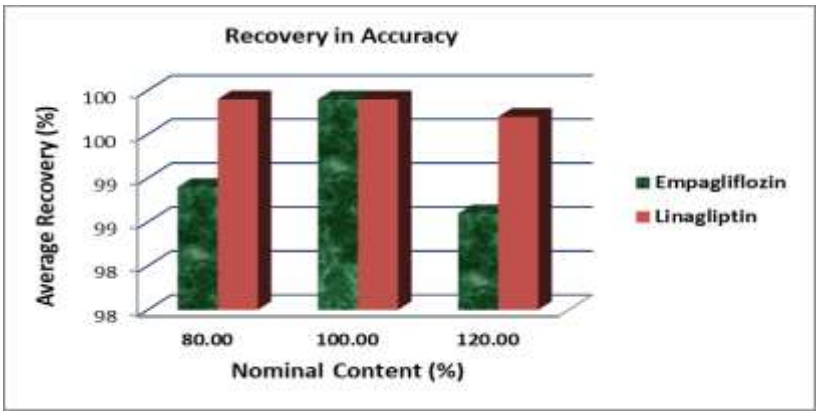

Figure 12. The Graphical assessment of the method's accuracy for determining Linagliptin and Empagliflozin in commercial formulations.

Precision

The degree of correspondence between the distribution of test results around the mean value and the individual test results are indicators of an analytical procedure's precision, and they are usually stated as the Relative Standard Deviation or Standard Deviation.

The entire process is repeated on several samples taken from the same homogenous bulk to achieve the results. Precision was assessed through measurements of repeatability, reproducibility, and intermediate precision.

Repeatability

Six replicates of a sample solution containing the target level of analyte were examined in order to assess repeatability. The sample solution was synthesized using the final method approach. To further ensure the testing's accuracy process, six distinct assays of Empagliflozin-L 25 mg/5 mg tablets were carried out in comparison to the standard. For each of the six tests, the Relative Standard Deviation (RSD) was computed.

Results are presented in Tables 4 and 5, with graphical representation provided in Figure 13.

Name	Weight of Empagliflozin sample (µg/mL)	Area / Abs Empagliflozin	Amount Found Empagliflozin (µg/mL)	% Recovery
Standard	100	3104979		
Sample -1	100	3108640	99.42	99.4
Sample -2	100	3098808	99.10	99.1
Sample -3	100	3107026	99.37	99.4
Sample -4	100	3108523	99.41	99.4
Sample -5	100	3099084	99.11	99.1
Sample -6	100	3106238	99.34	99.3
			Average	98.9
			Std. Deviation	0.22
			CI 95% ±	0.19
			%RSD	0.22

Table 4: Repeatability of empagliflozin determination by HPLC.

Name	Weight of Linagliptin sample (µg/mL)	Area / Abs Linagliptin	Amount Found Linagliptin (µg/mL)	% Recovery
Standard	20	444239		
Sample -1	20	445131	99.48	99.5
Sample -2	20	442769	98.95	99.0
Sample -3	20	444627	99.37	99.4
Sample -4	20	445657	99.60	99.6
Sample -5	20	443583	99.13	99.1
Sample -6	20	443322	99.07	99.1
			Average	99.3
			Std. Deviation	0.25
			CI 95% ±	0.22
			%RSD	0.25

Table 5: Repeatability in Linagliptin HPLC determination.

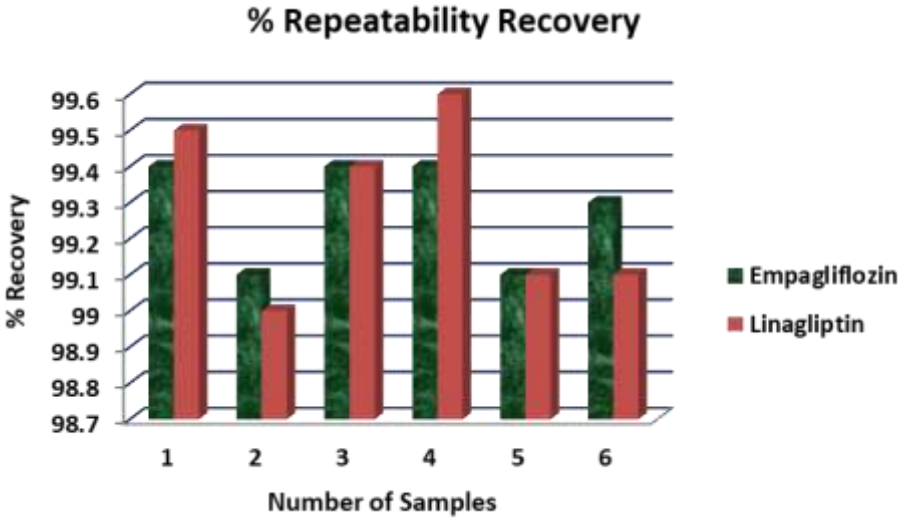

Figure 13: Repeatability of the procedure for determining the amounts of empagliflozin and linagliptin in commercial formulations

Reproducibility:

The means of the drug substance's peak area were obtained by using six replicates of the standard solution on the same HPLC apparatus as well as their corresponding standard deviations were analysed to assess the technique's reproducibility. The preparation process for the standard solution was the same as previously mentioned.

Results are summarized in Table 6, with graphical representation provided in Figure 14.

Standard Solution	Peak Area Empagliflozin	Peak Area Linagliptin
Injection 1	3112485	445730
Injection 2	3112312	446868
Injection 3	3111528	446130
Injection 4	3110993	446928
Injection 5	3111448	445311
Injection 6	3109074	446325
Mean	3111307	446215
Standard Deviation	1228.9 %	633.48 %
% RSD	0.039 %	0.141 %

Table 6: The method's reproducibility in determining the amounts of linagliptin and empagliflozin in commercial formulations

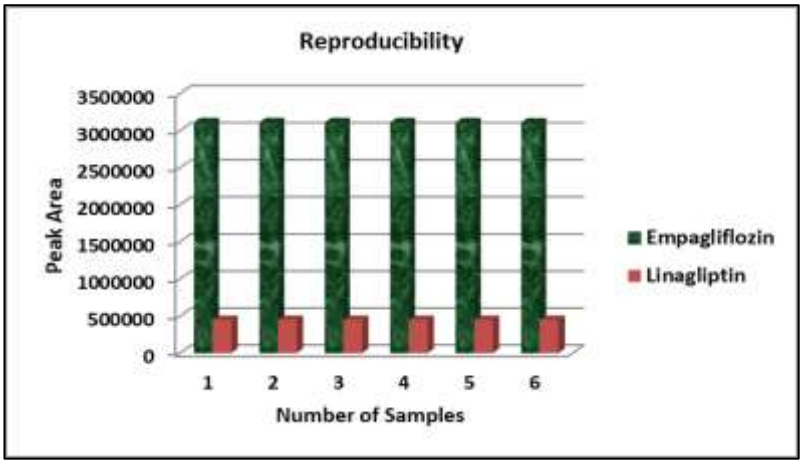

Figure 14: The method's reproducibility in determining the amounts of empagliflozin and linagliptin.

Intermediate Precision:

The same analyst evaluated intermediate precision—a measure of within-laboratory variation—using two distinct HPLC systems on separate days. For every HPLC system, the means and relative standard deviations of the drug substances' percentages were computed. The same protocol that was outlined for sample preparation was used to prepare the samples. Table 7 provides more specific results.

Parameter	Assay Empagliflozin	Assay Linagliptin
HPLC 1	99.73 %	99.37 %
HPLC 2	100.33 %	100.38 %
Mean	100.03 %	99.88 %
Standard Deviation	0.424	0.714
% RSD	0.424 %	0.715 %
HPLC 1	99.66 %	99.41 %
HPLC 2	99.67 %	99.83 %
Mean	99.66 %	99.62 %
Standard Deviation	0.007	0.296
% RSD	0.007 %	0.298 %

Table 7: The method for determining the amounts of linagliptin and empagliflozin in commercial formulations has an intermediate level of precision.

Robustness

An analytical method is said to be robust if it can continue to function normally in the face of small, deliberate changes in the experimental parameters. This indicates the method is suitable and reliable for regular use.

Every time one of the parameters was changed during these tests while the others stayed the same, the retention time of the peak was recorded. Table 8 shows that minor differences in methodology variables did not significantly impact the procedures.

Change in chromatographic conditions	% RSD of Empagliflozin	% RSD of Linagliptin
Mobile phase 645B : 355A	0.000	0.132
Mobile phase 650B : 350A	0.011	0.023
Mobile phase 655B : 345A	0.122	0.013
Flow rate 0.90 mL/min	0.006	0.022
Flow rate 1.0 mL/min	0.006	0.026
Flow rate 1.10 mL/min	0.000	0.132
Column oven Temperature 25 °C	0.055	0.113
Column oven Temperature 30°C	0.011	0.026
Column oven Temperature 35°C	0.022	0.058

Table 8: How minor changes to the assay setup affect the suggested HPLC method's analytical performance in detecting empagliflozin and linagliptin.

Conclusion:

Using easily obtained materials, the suggested high-performance liquid chromatography method for identifying linagliptin and empagliflozin is straightforward, economical, and ecologically benign.

With a 5 µm particle size (250 × 4.6 mm), an ODS – 3V column was used for the chromatographic conditions. The mobile phase for the PDA detector was a 650:350 ratio of acetonitrile to buffer (ammonium acetate). 12.0 minutes was the run time in total. Empagliflozin and Linagliptin had ideal wavelengths for detection of 210 nm and 290 nm, respectively. The optimal flow rate was 1.00 mL/min. Empagliflozin demonstrated a linear calibration curve with a regression coefficient of 0.9999 over the concentration range of 20–120 µg/mL, while linagliptin demonstrated a linear calibration curve with a correlation coefficient of 0.9999 over the range of 4.0–24.0 µg/mL. Empagliflozin and Linagliptin were found to have respective LODs of 1.48 µg/mL and 0.313 µg/mL. Empagliflozin's LOQ was 4.48 µg/mL, while linagliptin's was 0.949 µg/mL. While Linagliptin displayed an accuracy range of 99.68 – 99.90 percent, empagliflozin demonstrated an accuracy range of 98.60 – 100.12 percent. These findings suggest that routine examination of linagliptin and empagliflozin in pharmaceutical preparations can be successfully performed using the suggested approach.

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