ISSN: 2663-2187

Patil Nilesh I /Afr.J.Bio.Sc.6(si2) (2024)

https://doi.org/10.48047/AFJBS.6.si2.2024.5990-6009

Bioanalytical stability indicating method development and validation for the estimation of Anti diabetic drugs in human plasma by using RP-HPLC Method

Patil Nilesh I.[1*], Dr. Gupta Revathi A.[2]

[1]Research scholar Dr. A. P. J. Abdul Kalam University Indore – Dewas bypass road Village Arandia P O Vijay nagar Indore. MP.

[2]Professor and Dean Faculty of Pharmacy Dr. A. P. J. Abdul Kalam University Indore – Dewas bypass road Village Arandia P O Vijay nagar Indore. MP

*** Corresponding Author**
Mr. Nilesh I Patil
Research scholar Dr. A. P. J. Abdul Kalam University Indore – Dewas bypass road Village Arandia P O Vijay nagar Indore.MP.Mail id nileshwarpatil89@gmail.com

Volume 6 issue si2 2024

Received:15May2024

Accepted:10June2024

doi:10.48047/AFJBS.6.

si2. 2024. 5990-6009

ABSTRACT
The primary aim of this work is to develop a quick, sensitive, and selective RP-HPLC method with straightforward extraction processes, minimal solvent and biological fluid use, and a rapid turnaround time. We have developed and validated an RP-HPLC method for estimating Empagliflozin and Linagliptin in human plasma. Using an Agilent C18 column (50µm, 4.6×250 mm) as the stationary phase, with a mobile phase of methanol and 0.1% ortho phosphoric acid in water (45:55, pH 2.5) at a 0.7 mL/min flow rate, and DAD detection at 248 nm, the method proved effective. It showed linearity over a concentration range of 10-50 µg/mL for Empagliflozin and 5-25 µg/mL for Linagliptin, with retention times of 4.186 min and 6.532 min, respectively. The method's specificity, precision, accuracy, and robustness were confirmed according to ICH guidelines. The limits of quantification (LOQ) were 0.45 µg/mL for Empagliflozin and 0.31 µg/mL for Linagliptin, while the limits of detection (LOD) were 0.15 µg/mL and 0.10 µg/mL, respectively. Stability tests showed the drugs remained stable through three freeze-thaw cycles. This RP-HPLC method is simple, reliable, precise, accurate, sensitive, and selective, making it suitable for routine quantitative analysis in pharmaceutical dosage forms, as well as for therapeutic drug monitoring, bioequivalence, bioavailability, pharmacokinetic, and toxicology studies of antidiabetic drugs.
Keywords: *Bio-analytical method, RP-HPLC, Validation, Empagliflozin, Linagliptin*

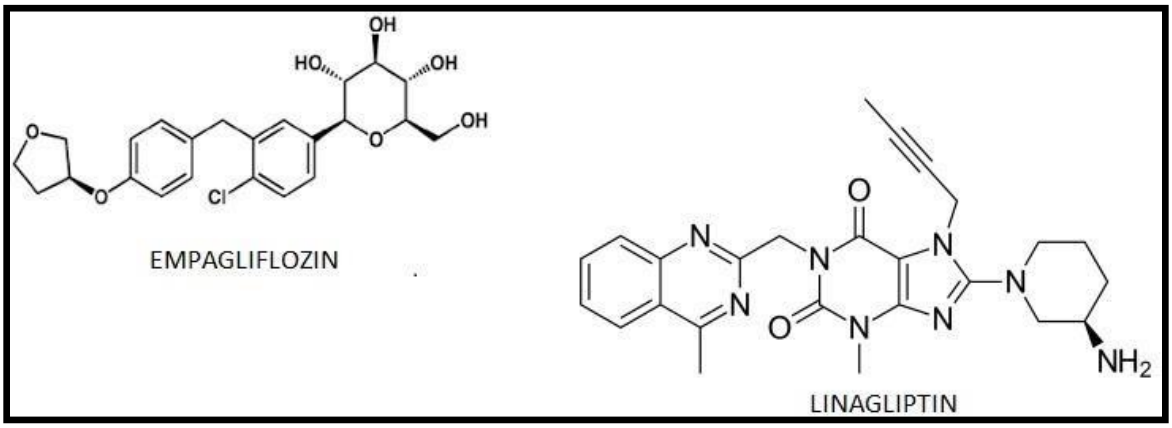
INTRODUCTION

Type 2 diabetes is a chronic, progressive, and multifactorial metabolic disease defined by the presence of chronic hyperglycemia [1]. Gastrointestinal incretin deficiency/resistance, increased renal glucose reabsorption, α-cell hyper glucagonemia, decreased pancreatic insulin secretion, increased peripheral insulin resistance, impaired lipolysis, increased hepatic glucose production, and neurotransmitter dysfunction make up the clinical pathological factors.[2]. Obesity and an irregular lifestyle are the main causes of the significant rise in the number of type 2 diabetic patients. Glycosylated hemoglobin (HbA1c) is positively correlated with a 38% increased risk of a macrovascular event in patients with type 2 diabetes for every 1% increase.[3].For patients with type 2 diabetes, metformin is generally the most prescribed medication due to its effectiveness, safety, weight neutrality, and affordability [4]. Some patients, however, either show signs of metformin intolerance or contraindication, or are unable to reach glycemic targets with metformin alone due to a progressive deterioration of β-cell function. For the treatment of type 2 diabetes, combination therapy (dual or triple) using various anti-diabetic medication mechanisms is eventually started. [5]. A recent study showed that glycemic control was improved more when triple therapy—an SGLT2 inhibitor and a DPP-4 inhibitor—was added to baseline metformin therapy. Developed to treat type 2 diabetes, Empagliflozin (Jardiance®) is a sodium glucose co-transporter-2 (SGLT2) inhibitor that was approved in the US and EU in 2014 [6].

Oral tablets containing linagliptin and empagliflozin are marketed for the management of type 2 diabetes and cardiovascular risk. As an inhibitor of sodium glucose cotransporter-2 (SGLT-2) empagliflozin (EMPA) helps adult patients with type 2 diabetes better control their blood sugar levels. Because SGLT-2 inhibition lowers renal absorption and the renal glucose threshold, which in turn raises glucose secretion and lowers blood pressure and hyperglycemia, SGLT-2 co-transporters reabsorb glucose from the glomerular filtrate and the renal glucose-producing substance action. The competitive, reversible DPP-4 inhibitor linagliptin (LINA) inhibits the breakdown of both GLP-1 and the glucose-dependent insulin tropic polypeptide (GIP). GLP-1 and GIP inhibit the release of glucagon from pancreatic beta cells while stimulating the release of insulin from these cells. Together, these outcomes result in less hepatic breakdown of glycogen and more insulin being released in response to glucose. It is used clinically to treat type 2 diabetes mellitus as a supplement to diet and exercise, frequently in conjunction with other medication therapies. [7]

Description of Empagliflozin and Linagliptin

Figure 1: Chemical structure of Empagliflozin and Linagliptin



Chemically speaking EMPA is 1chloro-4-(glucopyranos-1-yl)-2-(4-(tetrahydrofuran-3-yloxy)benzyl)benzeneand having empirical formula is C23H27ClO7 with molecular weight 450.91 g/mole .Chemically LINA is (R)-8-(3-aminopiperidin-1-yl)-7-but-2-ynyl-3 methyl-1-(4-methyl quinazolin-2-ylmethyl)-3,7-dihydro-purine-2,6-dione and having empirical formula is C25H28N8O2 with molecular weight 472.5422 g/mole. Linagliptin increases bodily chemicals that cause the pancreas to release more insulin, which aids in blood sugar regulation. In addition, it notifies the liver to cease synthesizing glucose when the blood sugar level is excessive. [8-12]

MATERIALS AND METHODS

Procured pure standards of Empagliflozin (EMPA) and Linagliptin (LINA) from R.S I T C Jalgaon. Purchased Marketed tablet formulation of Brand name as AJADUO, which contained 10 mg of EMPA and 5 mg of LINA from pharmacy shop sold by it. The following products were bought from Merck ltd methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), orthophosphoric acid (AR grade), and triethylamine (AR grade).

Instrumentation:

Chromatographic measurements were made on AGILET (1100) HPLC having detector (G-13148 with DAD source in the range 245 nm with double reciprocating plunger pump with constant flow and pressure delivery. The mobile phase was degassed by using Ultrasonicator (3.5L100) Ultrasonics electronic instrument Ultra sonic bath. The UV spectrum was recorded using a UV-Visible spectrophotometer (Analytical Technologies Limited, Japan (Model UV 2080) – software UV analyst.

Mobile phase selection:

The main requirement of the mobile phase is that it has to dissolve the analytes up to the concentration suitable for detection. The mobile phase absorbance should usually be less than 0.5 at the wavelength used for detection. When the absorbance of the mobile phase exceeds a value of about 1.0 the detector may become unusable. Hence the mobile phase

suitable for samples is selected by performing trials with different ratios of the mobile phase.

Chromatographic condition:

HPLC: Agilet (1100)
Software: Chemstation10.01
Stationary phase: C18 column (Agilent)
Mobile phase: Solvent A – Methanol Solvent B – 0.1%OPA
Solvent ratio: 45: 55 (A: B)
Detection Wavelength: 248 nm
Flow rate: .0.7 ml/min
Temperature: Ambient
Sample size: 20 µl

Preparation of Mobile phase:

45.0 % of methanol and 55.0 % of 0.1%Orthophosperic acid were combined to create the mobile phase.After passing through a 0.42 µ membrane filter, this mobile phase underwent 15 minutes of ultrasonication.

Preparation of Sample Solution:

Transferred about 10 mg of Empa and 5 mg of Lina standard into a 25 mL volumetric flask, added approximately 10 mL of diluent, (methanol) then vertically shacked for 30 min then centrifuged at 5000 rpm for 1 hr. Then it was filtrated using membrane filters to get clear organic solution. Then it was filled in to the sample vials of HPLC and loaded on to HPLC for Run.(Empa and Lina concentrations are 500µg/ml Lina&1000 µg/ml Empa indicate as Stock I Solution.

The solutions were brought up to volume with mobile phase using an A-grade bulb pipette into 10 ml volumetric flasks, giving final concentrations of 5-25µg/mL for Lina and 10-50µg/mL for Empa.

Preparation of plasma sample:

At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. 2.0 mL of sample was pipetted into 10 ml centrifuge tube with this 10mg of Empagliflozin solution (1000 µg/ml) and 5 mg of Linagliptin solution (500µg/mL) was added. The resulting solution was vortexed for 10 minutes and centrifuged at 5000 r/min for 10 min. 5000 RPM for effective phase separation. organic layer was pipette out into a separate tube and evaporated to dryness. The residue was then reconstituted with 10mL mobile phase and subjected to chromatographic analysis.

Method Development:

The mobile phase consisting of methanol and 0.1% Orthophosperic acid in varying proportions and change in pH was tried and finally ratio of 45:55 (pH-2.5 adjusted with orthophosphoric acid) was selected because it was found to give good separation for the peaks of Empa(Rt-4.186 min) and Lina (Rt-6.532 min) respectively as shown in the **figure**

7. In addition to this, UV spectra of individual drugs were recorded at the wavelength range from 200 to 400 nm and the response for optimization was compared. The choice of wavelength 248 nm was considered satisfactory, permitting the detection of drugs with adequate.

RESULTS AND DISCUSSION

Method Validation:

The method was validated in accordance with USFDA guidelines and EMEA guidelines. [13-14]

A Bioanalytical RP-HPLC method was developed for the Empa and Linagliptin. The chromatographic conditions were stabilized in order to provide a good performance of the assay. The standard solutions were prepared and chromatograms were recorded. The study proposes a method for the determination of Empa and Lina combination in human plasma by using RP-HPLC.

Selection of wavelength for 20 µg/mL Linagliptin in Methanol (0.2ml in 10ml MeOH)

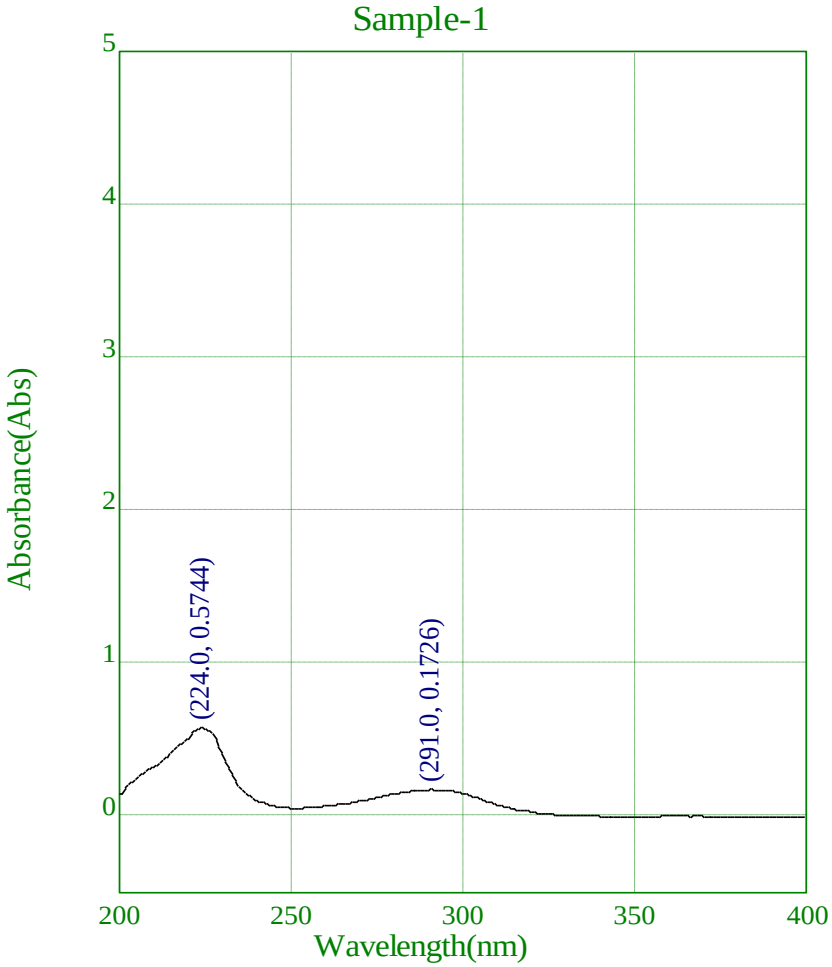


Figure 2: Uv spectrum of Linagliflozin

Selection of wavelength for 20 µg/mL Empa in Methanol

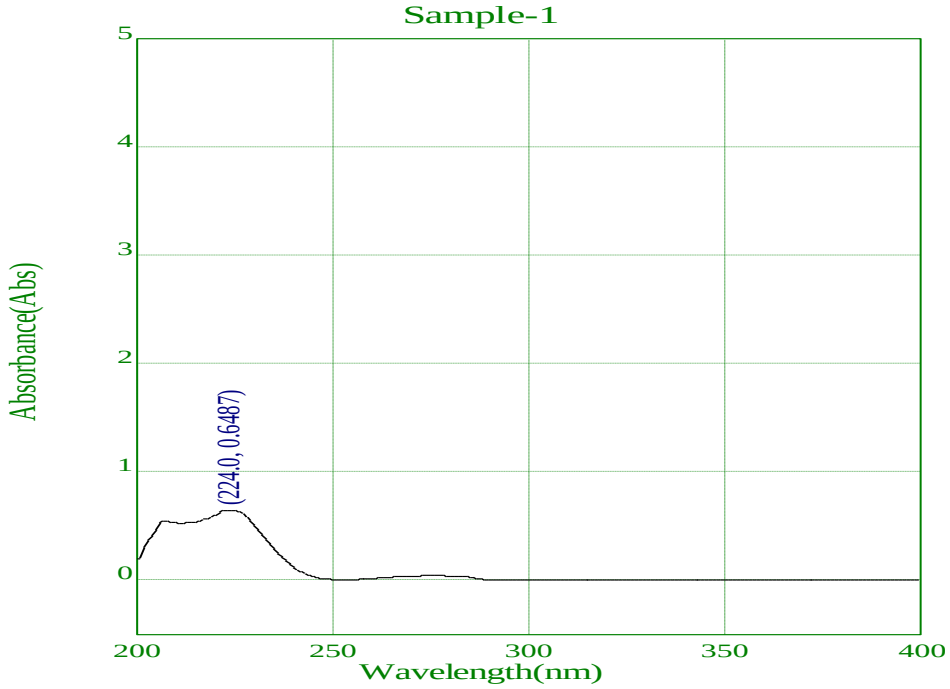


Figure 3: Uv spectrum of Empagliflozin

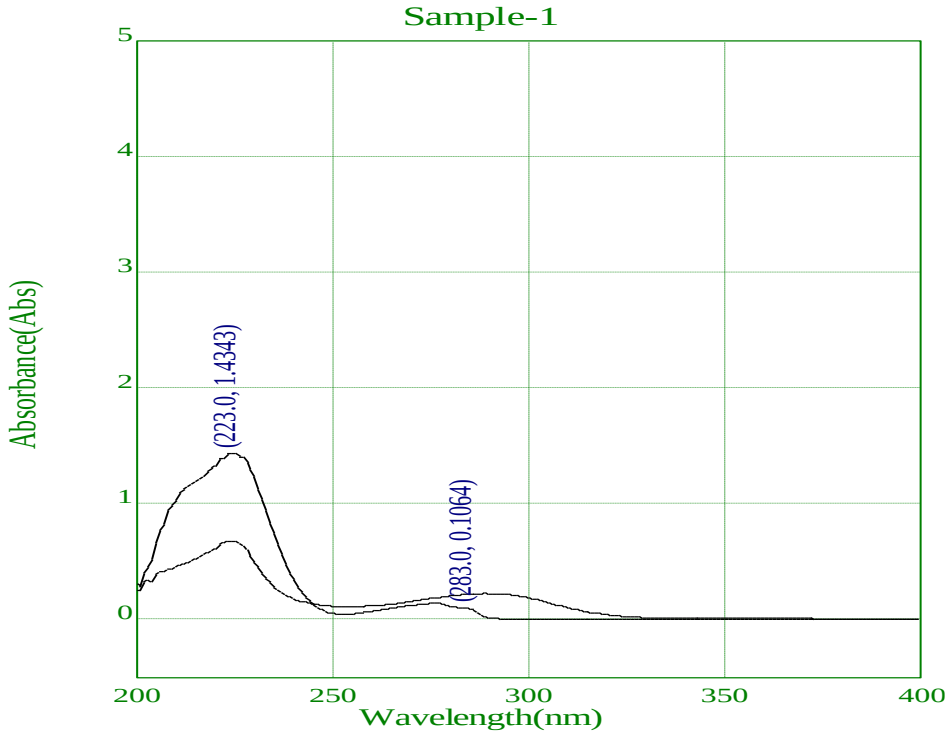


Figure 4: Isobestic Point of Combination drug at 248 nm

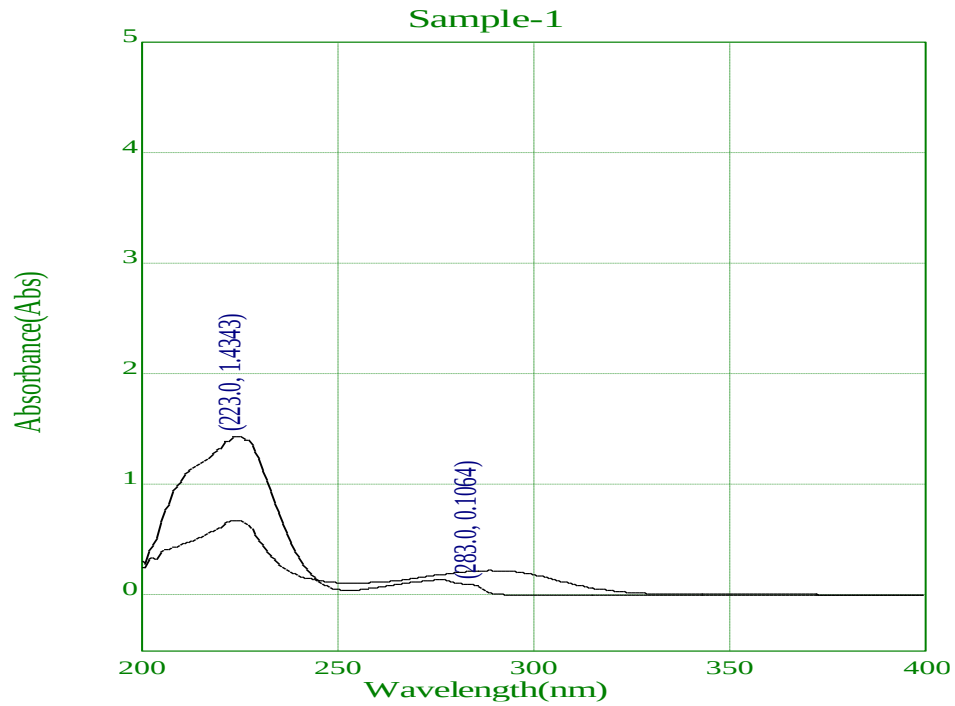


Figure 5: Second Isosbestic Point of Combination drug at 245 nm

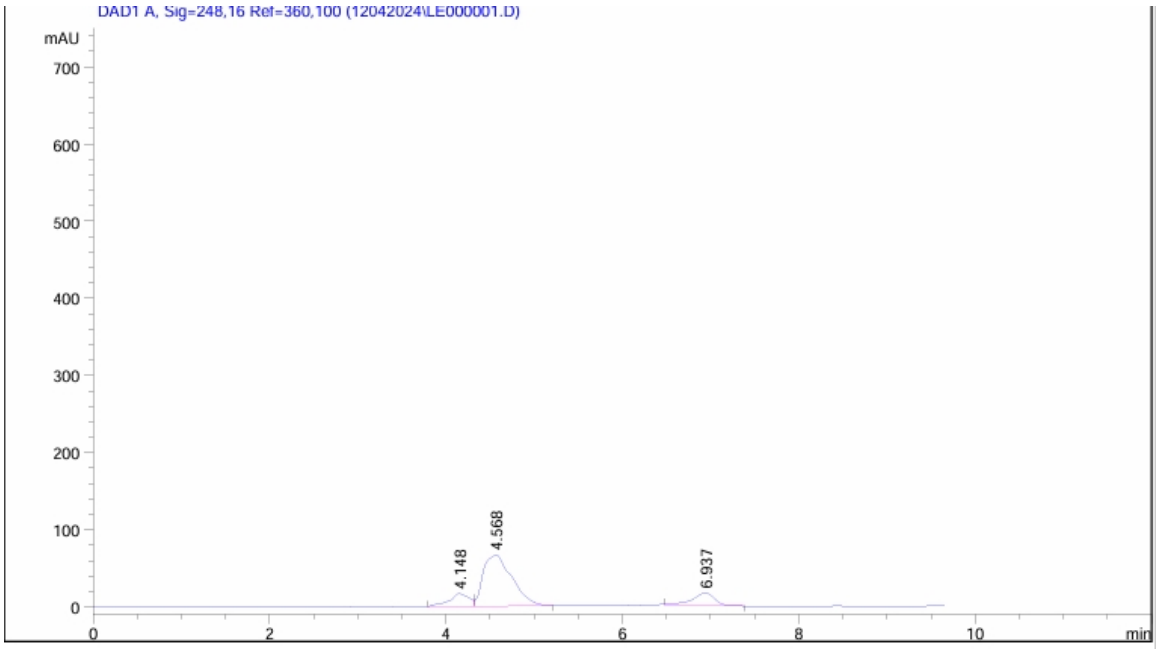


Figure 6 :Representative Chromatogram of Blank Plasma

1)1. Specificity (selectivity)

The ability of an analytical technique to identify and measure the analyte in the presence of other sample constituents is known as selectivity. The specificity of method was performed by comparing the chromatogram of blank, standard and sample. The retention time found is represented in below chromatogram.

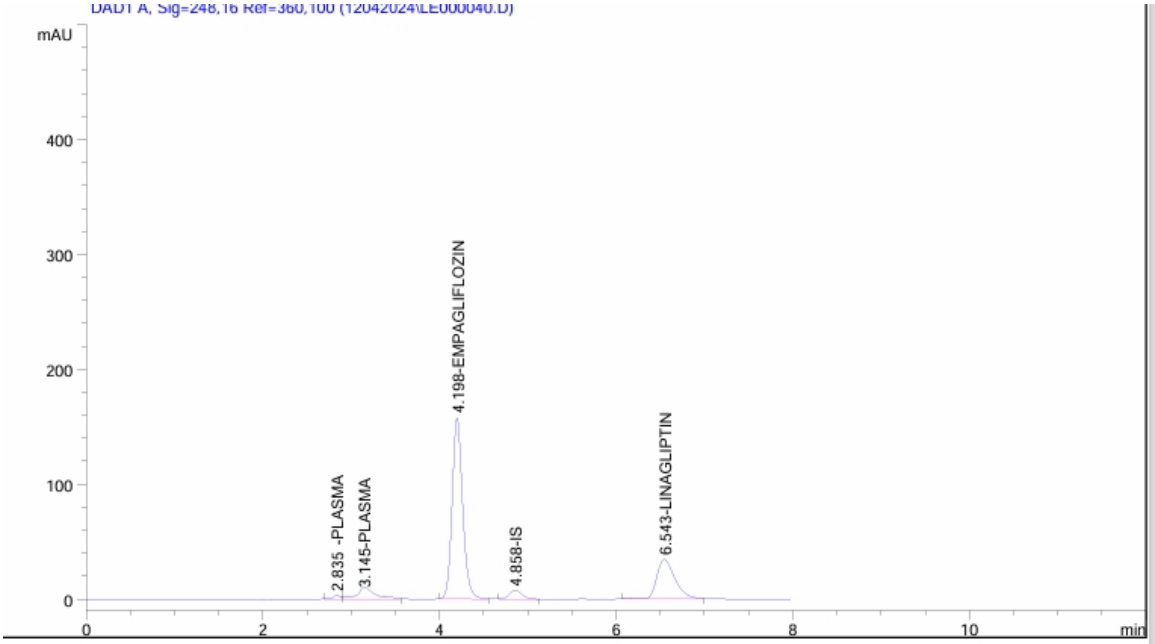


Figure 7: Specificity Chromatogram of Empa and Lina with Plasma.

Table 1: Specificity result Empa and Linagliptin

Sr No	Solution	Area	%RSD	Retention time
1	Blank	0	0	0
2	20µg/mLEmpagliflozin	1089.42	0.129	2.224
3	10µg/mL Linagliptin	408.02	0.274	4.374

2. Accuracy:

The degree to which the mean test results produced by the method closely resemble the actual value (concentration) of the analyte is known as the analytical method's accuracy. Recovery studies were used to calculate accuracy. For EMPA, it was discovered that the mean percentage recoveries values ranged from 99.70 across three levels. The accuracy of the developed method is indicated by the percentage of recoveries falling within the given limits

Table 2: Accuracy result for Empagliflozin

% Cons at specified level	Area	Amount added(mg)	Amount Founded (mg)	% Recovery
10 µg/mL	985.83	80	17.92	99.28
10 µg/mL	1091.0100	100	19.92341	99.28

10 µg/mL	1201.59	120	22.0249	100.56

*Average of three determinations

Accuracy Result for Linagliptin

It was found that the mean percentage recoveries values for the three levels ranged from 99.8 for Linagliptin, respectively. The accuracy of the developed method is indicated by the percentage of recoveries falling within the given limits.

Table 3: Accuracy Result for Linagliptin

% Cons at specified level	**Area**	**Amount added(mg)**	**Amount Founded (mg)**	**% Recovery**
5 µg/mL	372.07000	4	9.03	100.37
5 µg/mL	409.2700	5	9.981	99.66
5 µg/mL	447.08	6	10.94688	99.37

*Average of three determinations

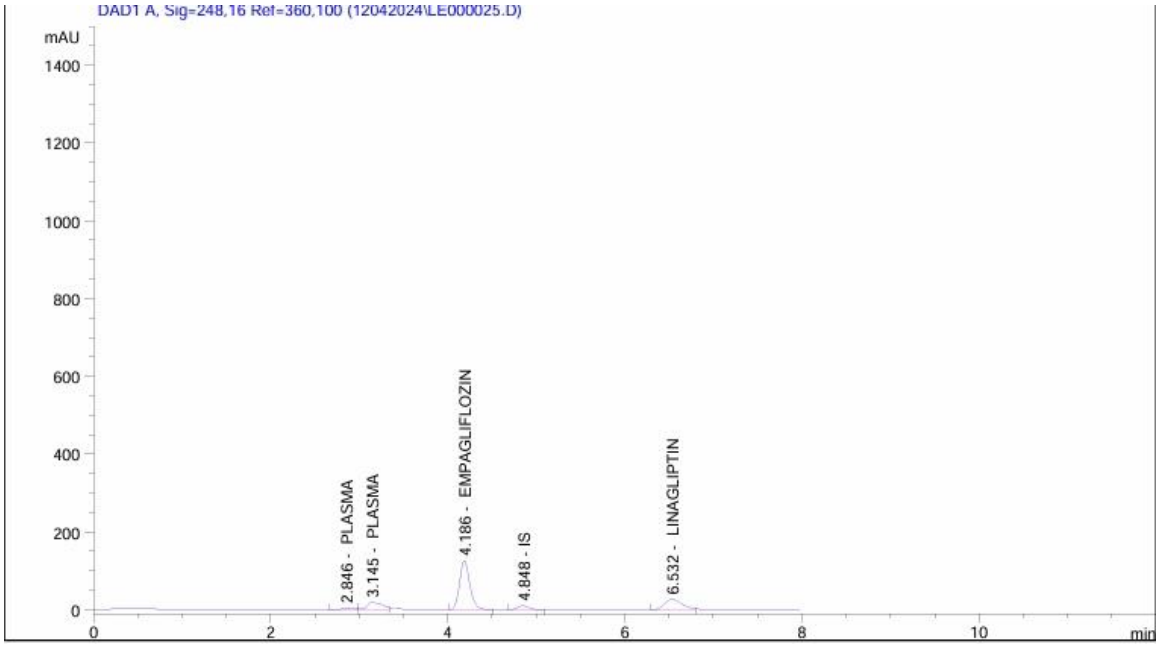


Figure 8: Representative Accuracy Chromatogram of Empa and Lina with Plasma.

3**. Precision**:

When an analytical procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix, precision refers to the closeness of individual measures of an analyte. The system, method, and intermediate precision study's percentage RSD was well within the bounds (<2%), demonstrating the precision of the procedure.

Table 4: Precision Results for Empagliflozin

Injection Intraday	**Area of Empagliflozin**	**SD**	**%RSD**
20µg/mL	1087.06	5.19	0.476
30 µg/mL	1619.24	3.56	0.22
40 µg/mL	2120.48	3.00	0.142
Interday	**Area of Empagliflozin**	**SD**	**%RSD**
20µg/mL	1093.34	1.49	0.14
30 µg/mL	1613.83	0.83	0.05
40 µg/mL	2120.43	3.55	0.17

*****Average of three determinations, %RSD: Percentage relative standard deviation

Table 5: Precession result for Linagliptin

Injection Intraday	**Area of Linagliptin**	**SD**	**%RSD**
10 µg/mL	406.9	1.83	0.62
15µg/mL	600.88	1.25	0.33
20 µg/mL	798.34	1.21	0.26
Interday	**Area of Linagliptin**	**SD**	**%RSD**
10 µg/mL	405	0.08	0.02
15 µg/mL	601.4	0.64	0.11
20 µg/mL	801.7	1.03	0.13

*****Average of three determinations, %RSD: Percentage relative standard deviation

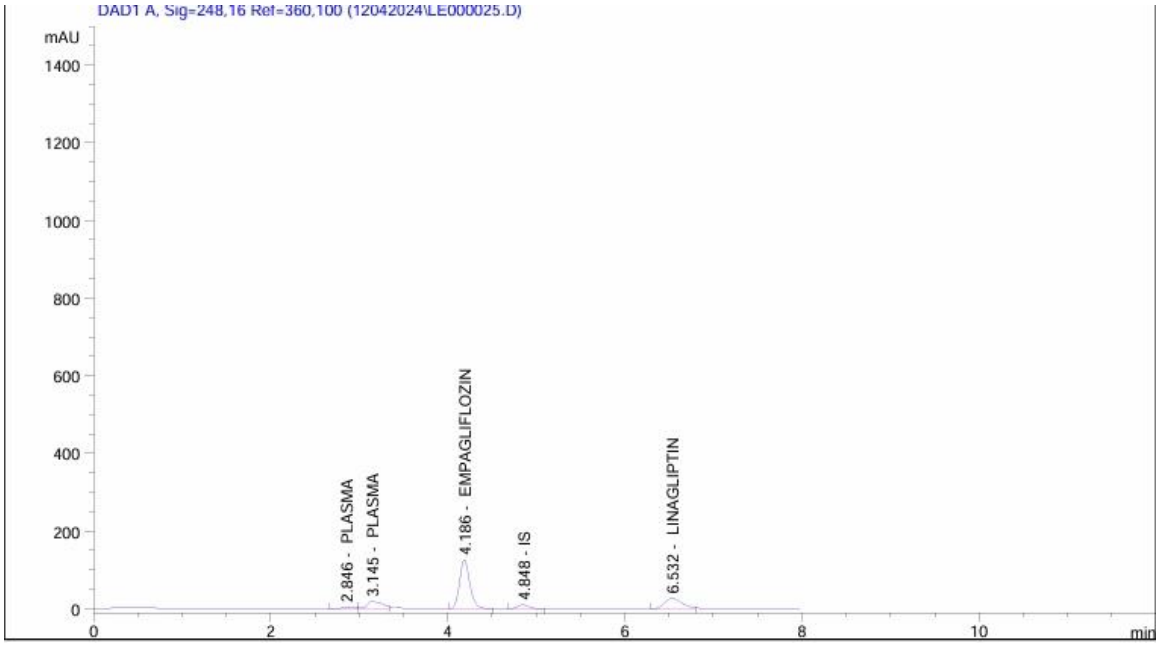


Figure 9: Representative Precision Chromatogram of Empa and Lina with Plasma.

4. **Repatability for Empagliflozin**

Table 6: Repeatability of Empagliflozin

Conc	Area of Empagliflozin	SD	%RSD
20 µg/mL	1093.04	0.69	0.06

Repatabilityof Linagliptin

Table 7: Repeatability of Linagliptin

Conc	Area of Linagliptin	SD	%RSD
10 µg/mL	405.84	1.05	0.26

5. Linearity

A) Empagliflozin

The calibration data was analyzed using least-squares linear regression to determine linearity. For Empa, the calibration plots showed linearity in the concentration range of 10–50 µg/ml. plotting peak areas against corresponding concentrations was done, and the resulting curves were subjected to linear regression analysis. The linear curve of Empa was shown in **Figure 10** respectively. The linear regression equation obtained was Y=52.62x+42.64 for Empa. With correlation coefficient 0.999respectively. The results of linearity are shown in Table 8.

Table 8: Linearity result for Empagliflozin

Sr No	Linearity Level	Concentration	Area
1	I	10 µg/mL	591.82
2	II	20 µg/mL	1088.09
3	III	30 µg/mL	42.64
4	IV	40 µg/mL	52.62
5	V	50 µg/mL	2659.10
Correlation coefficient	R^2= 0.999		

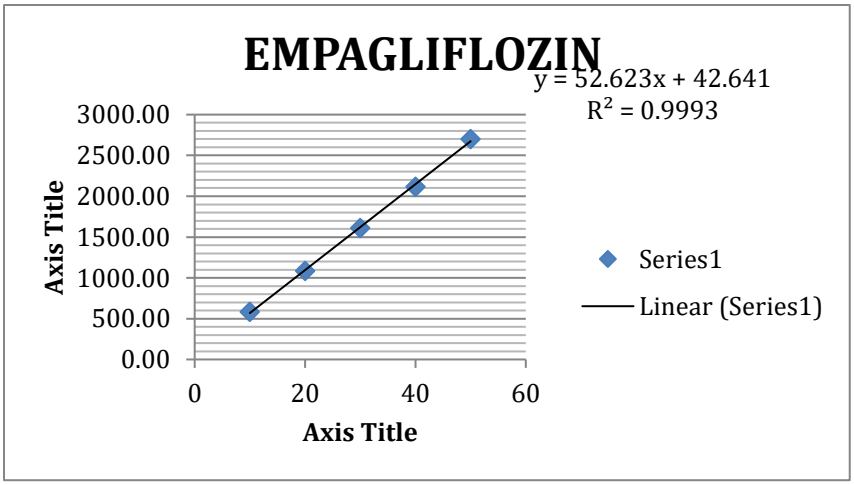


Figure 10: Representative linearity of Empagliflozin

B) Linearity result for Linagliptin

The calibration data was analyzed using least-squares linear regression to determine linearity. The calibration plots for Lina showed a linear relationship in the concentration range of 5–25 µg/ml .Peak areas were plotted against the associated concentrations and the resulting curves were subjected to linear regression analysis. The linear curve of Linagliptin was shown in **Figure 118** respectively. The linear regression equation obtained was Y=39.16x+18.40 for Lina. With correlation coefficient 0.999 respectively. The results of linearity are shown in

Table 9: Linearity result for Linagliptin

Sr No	**Linearity Level**	**Concentration**	**Area**
1	I	5 µg/mL	217.64

2	II	10 µg/mL	409.52
3	III	15 µg/mL	60..55
4	IV	20 µg/mL	794.70
5	V	25 µg/mL	1004.26
Correlation coefficient R^2= 0.9993			

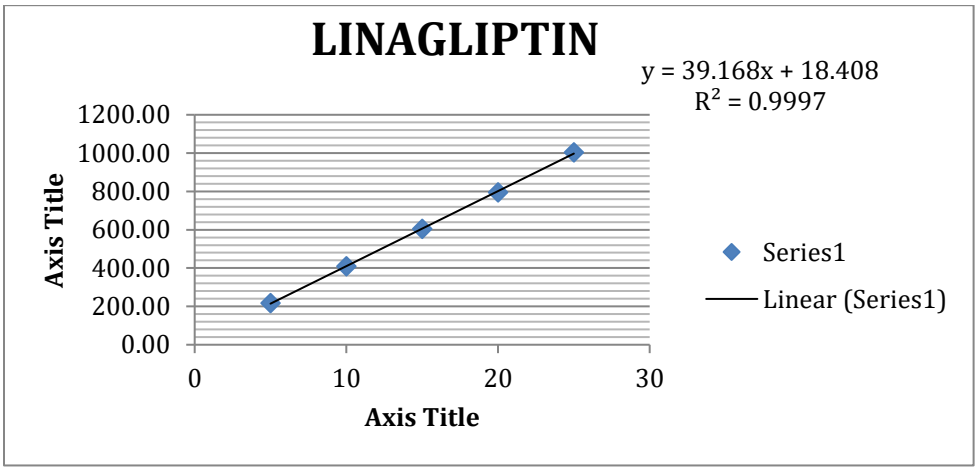


Figure 11: Representative linearity of Linagliptin

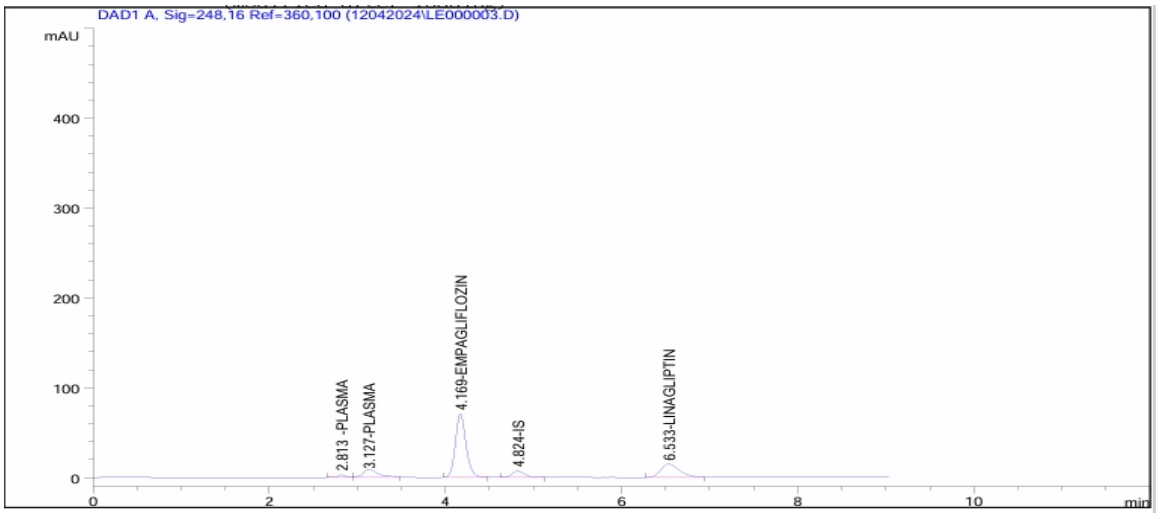


Figure 12: Representative Linearity Chromatogram of Empa and Lina with Plasma.

6. Robustness

Table 10: Robustness Study for Empagliflozin & Linagliptin

Condition		Empagliflozin		Linagliptin	
		SD	%RSD	SD	%RSD
Change in wavelength (210±1 nm)	247nm	0.33	0.02	2.24	0.21
	249nm	2.37	0.11	0.97	0.13
Change in flow rate (1.0±0.1 ml/min)	0.9ml	0.80	0.04	1.09	0.15
	0.8ml	1.43	0.06	12.09	1.27
Change in Mobile phase composition	44+56	0.60	0.03	0.66	0.08
	56+44	3.82	0.18	1.26	0.15

7. SystemSuitability parameters

Table 11: System Suitability parameters of drugs

Parameters	Empagliflozin	Linagliptin
Peak area	107.25749	986.714
Throtical plate	4074	5601
Retention time	4.429	2.222
Tailing factor	0.90	1.84

Assay of tablet:

The commercial formulation known by the brand name AJADUO was assayed by taking 20 tablets and triturating them. Calculating the average weight of 20 tablets, we found that it is 868 mg, with an average powder weight of 43.4 mg per tablet. Weigh the sample precisely, and then transfer the 43.4 mg equivalent weight into a 25 ml volumetric flask. Add roughly 10 milliliters of MEOH diluent (500 µg/mL linagliptin and 1000 µg/mL empagliflozin). Sonicate to fully dissolve and adjust volume with diluent.Blend thoroughly and pass through a 0.45 µm filter. Further pipette Use diluents to dilute 0.3 ml of the mentioned stock solution to the appropriate level in a 10-ml volumetric flask. (15µg/ml plus 30µg/ml). Assess the simple chromatogram of Lina and empa. The values of area were extrapolated from the calibration curve to determine the amounts of EMPA and LINA per tablet. Two iterations of the analysis process were conducted using tablet formulation. The

result was displayed in Table No. 11 for the tablet assay for %Label claim for %RSD Calculated.

Table 12: Analysis of marketed formulation.

Conc	**Area of Empagliflozin**	**SD**	**%RSD**
30 µg/mL	1600.53	6.131	0.394
Conc	**Area of Linagliptin**	**SD**	**%RSD**
15µg/mL	599.34	5.303	0.913

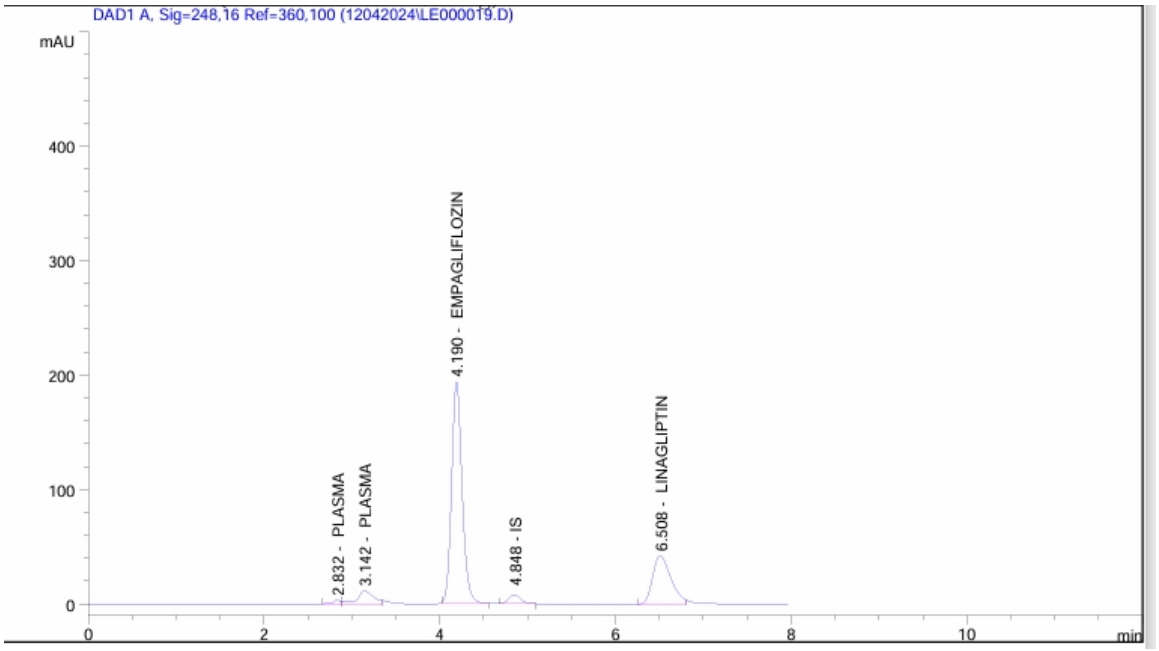


Figure no 13:Representative Assay Chromatogram of Empa and Lina with Plasma

Forced Degradation studies

Table 13: Degradation studies of Drugs.

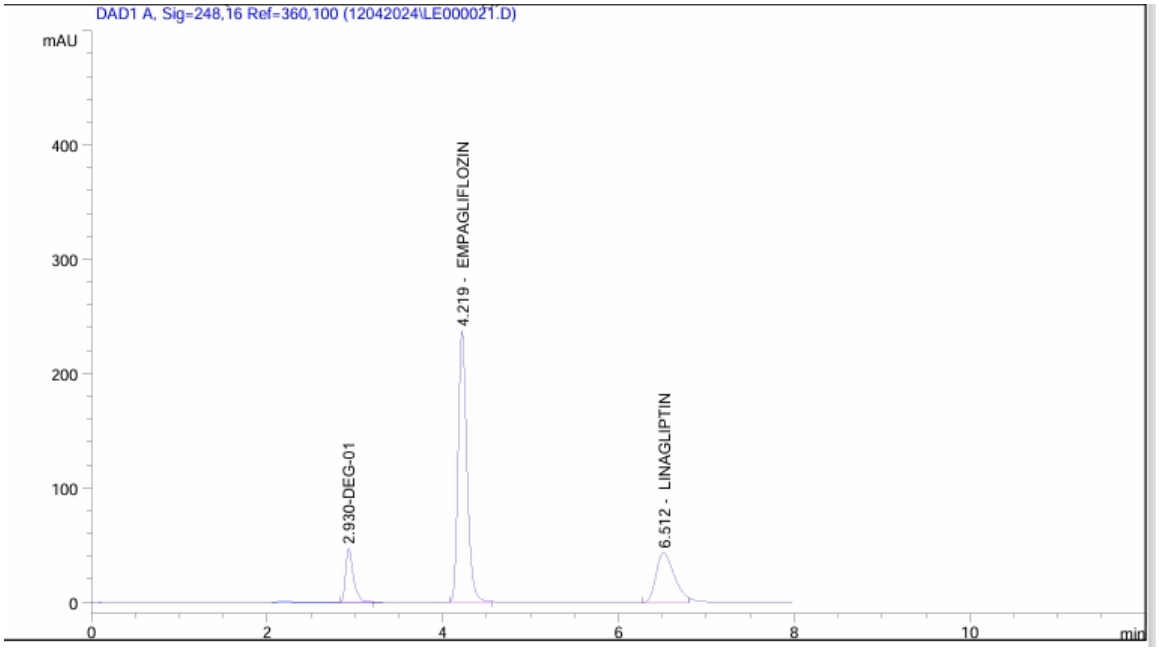
Sample name	**Standard Area**	**Area Degraded**		**Sample name**	**Standard area Area**	**Area degraded**	
Empagliflozin		**2Hrs**	**After 4Hrs**	**Linagliptin**		**2 Hrs**	**After 4Hrs**
Acid	1611.79529	1585.06	1576.82	Acid	603.978	582.68	576.04
Base	1611.79529	1580.45	1565.42	Base	603.978	585.39	572.12
H_2O_2	1611.79529	1485.62	1473.89	H_2O_2	603.978	573.79	535.20
Neutral	1611.79529	1596.62	1585.78	Neutral	603.978	596.70	593.81

Percentage degradation

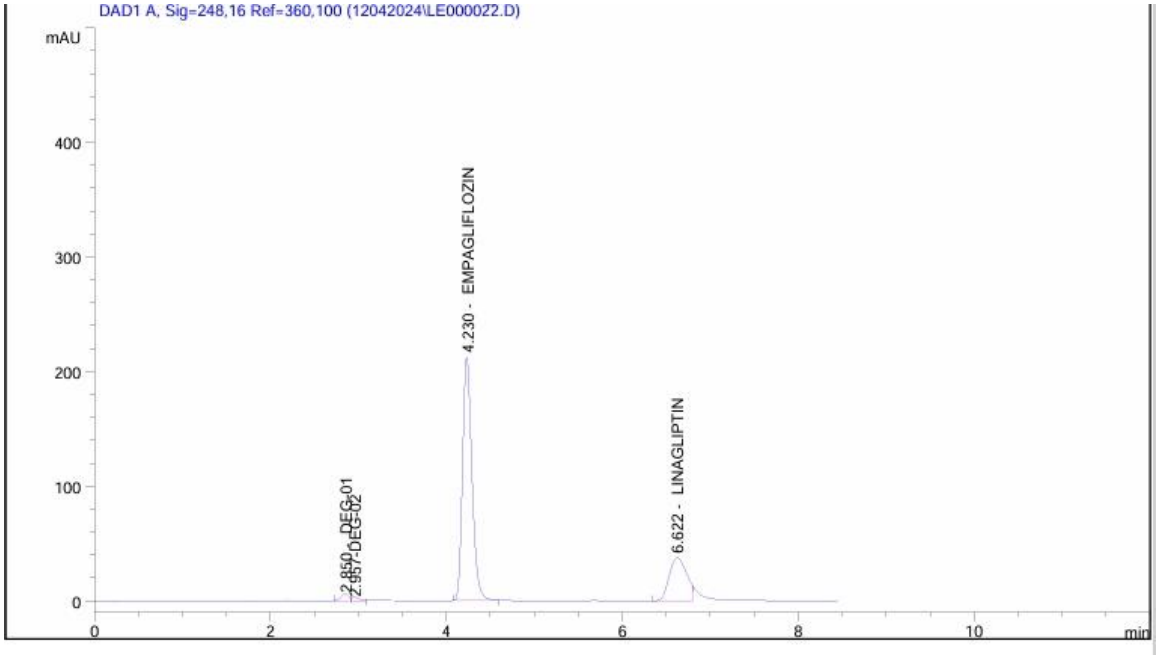
We are able to determine the sample's acidity, baseness, oxidation, and degradation based on these investigations. When a drug interacts with acid, it leads to primary degradation within the desired range in acid degradation. HCl or H2SO4 (0.1–1 M) is frequently used for acid analysis. In basic degradation when the drug interacts with base it produces primary degradation in the desirable range. For base analysis, NaOH or KOH (0.1–1 M) is widely used. In oxidative degradation hydrogen peroxide is widely used for oxidation degradation. Drug structure will allow selecting concentration and condition of oxidizing agent.

Table 14: Actual Percentage degradation

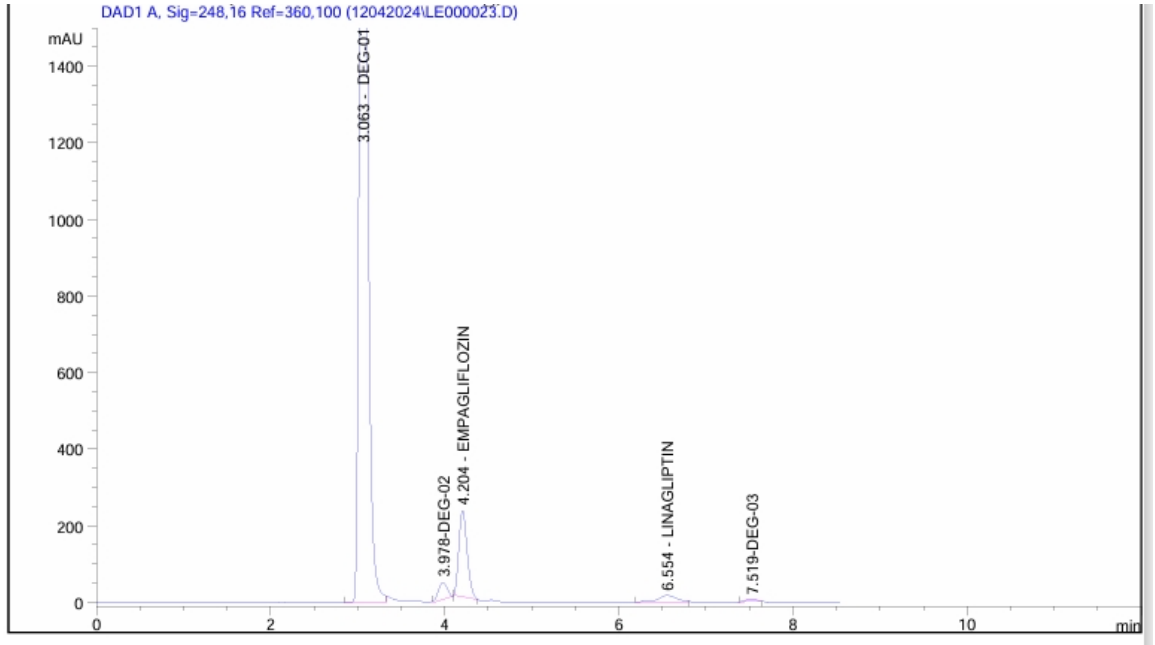
Actual % Degradation of Empagliflozin			**Actual % Degradation of Linagliptin**	
	2Hr	**After 4Hrs**	**2 Hr**	**After 4Hrs**
Acid	1.66	2.54	3.53	4.82
Base	1.94	2.88	3.08	5.27
H_2O_2	7.82	8.56	9.96	11.39
Neutral	0.94	0.61	1.020	1.07



Representative Chromatogram of Empa and Lina with Plasma in 0.1 N HCL



Representative Chromatogram of Empa and Lina with Plasma in 0.1 N NaOH



Representative Chromatogram of Empa and Lina with Plasma in 0.1 N H_2O_2 Report for validation Parameters

Table 15: Summary of validation parameter

Parameters	**Empagliflozin**	**Linagliptin**
Linearity Range(**µgm/ml)**	10-50	5-25
Slope	52.62	39.16
Intercept	42.64	18.4
Regression	0.999	0.999
Accuracy(%Recovery)	99.70%	99.80%
Precession(%RSD)	0.27	0.40
Assay (%)	98.69	98.90
LOD	0.15	0.10
LOQ	0.45	0.31

CONCLUSION

It is concluded that the developed bioanalytical method for estimating the combination of linagliptin and empagliflozin spiked in human plasma has not yet been reported. Additionally, it can quantify the combination of linagliptin and empagliflozin from human plasma samples that have been spiked. The technique can be used for bioavailability or bioequivalence studies and complies with the ICH Guidelines. Based on the information provided in this report, it can be said that the current approach is reliable for estimating the concentration of combined drugs in human plasma within the range of 10–50 µg/ml for EMPA and 5–25 µg/ml for LINA. The accuracy and precision are well within the allowed bounds in this concentration range. The anticipated recoveries for 80%, 100%, and 120% are observed with the current processing method.

Conflict of Interest: No conflict of Interest

Funding sources: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGEMENT

I am deeply grateful to my supervisor, Dr. Revathi A. Gupta, for her invaluable guidance and support throughout my Ph.D. journey. I also thank the Faculty of Pharmacy for the

opportunity to study at the Department of Pharmaceutical Sciences, Dr. A.P.J. Abdul Kalam University, Indore. Your support has been instrumental in my success.

REFERENCES

1. Garber, Alan J., et al. "Task force on the new comprehensive diabetes algorithm." *Endocrine practice: official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* 19.3 (2013): 536.
2. Roden, Michael, et al. "Empagliflozin monotherapy with sitagliptin as an active comparator in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, phase 3 trial." *The lancet Diabetes & endocrinology* 1.3 (2013): 208-219.
3. Garber, Alan J., et al. "Task force on the new comprehensive diabetes algorithm." *Endocrine practice: official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* 19.3 (2013): 536.
4. Friedrich, Christian, et al. "A randomized, open-label, crossover study to evaluate the pharmacokinetics of empagliflozin and linagliptin after coadministration in healthy male volunteers." *Clinical therapeutics* 35.1 (2013): A33-A42.
[5] Elmasry, Manal S., et al. "Flourimetric study on antidiabetic combined drugs; empagliflozin and linagliptin in their pharmaceutical formulation and human plasma." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 248 (2021): 119258.
[6] Padhi, Santwana, Amit Kumar Nayak, and Anindita Behera. "Type II diabetes mellitus: a review on recent drug based therapeutics." *Biomedicine & Pharmacotherapy* 131 (2020): 110708.
[7] Tripathi KD, Essentials of Medical Pharmacology.,5 [sup] Th Edn, New Delhi, Jaypee Brothers Medical publisher (P)Ltd778-9(2008) .
[8]Drugbank,Empagliflozin,https://www.drugbank.ca/drugs/DB09038Accessed 9July 2019
[9] Song, Ruisheng. "Mechanism of metformin: a tale of two sites." *Diabetes care* 39.2 (2016): 187-189.
[10] Grempler, R., et al. "Empagliflozin, a novel selective sodium glucose cotransporter-2 (SGLT-2) inhibitor: characterisation and comparison with other SGLT-2 inhibitors." *Diabetes, Obesity and Metabolism* 14.1 (2012): 83-90.
[11] Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, Malanda B. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract. 2018 Apr; 138:271-281. doi: 10.1016/j.diabres.2018.02.023. Epub 2018 Feb 26. PMID: 29496507.
[12] Unade T, Pawar. A New validated stability-indicating RP-HPLC method for the simultaneous determination of metformin hydrochloride, linagliptin and empagliflozin in bulk and pharmaceutical dosage forms. International Journal of Applied Pharmaceutics: 68-76.

[13]http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf
[14] Pandya RH, Rathod R, Maheswari DG. Bioanalytical method development and validation for simultaneous determination of linagliptin and metformin drugs in human plasma by RP-HPLC method. Pharmacophore. 2014 Mar 1; 5 (2):202-18.