DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF EMPAGLIFLOZIN, LINAGLIPTIN AND METFORMIN.

Kalpesh C Raka[1]*, Dr. Kavita R Loksh[2]

Abstract:
Analytical method development and validation are the continuous and inter-dependent task associated with the research and development, quality control and quality assurance departments. The analytical method development is the need of pharmaceutical industries. The development of methods usually requires the collection of method specifications and the decision on the type of instrumentation. Diabetes is a metabolic disorder with major complication associated with hyperglycemia. India is deemed as the world's capital of diabetes. The diabetic population in the country is close to hitting the alarming mark of 69.9 million by 2025 and 80 million by 2030. Combinations of various anti-diabetics are used for better sugar control. Nowadays various new combinations are in evaluated & are approved for better sugar control such as Empagliflozin, Linagliptin & Metformin hydrochloride.
This study describes the development of a rapid, precise, selective and sensitive reverse phase high-performance liquid chromatography method (RP–HPLC) for the quantitative simultaneous determination of Empagliflozin, Linagliptin and Metformin and validation as per International Council of Harmonization (ICH) guidelines. Initial identification of API was performed and is obaestic point was determined using UV spectrophotometry. In the present work, good chromatographic separation was achieved by isocratic method using aC_{18} (COSMOSIL)250 mm length x 4.6 mm internal diameter, 5 µm and a mobile phase consisting of Methanol: 0.1 % OPA Water (30:70)at a flow rate of 0.7 ml/min. G-13148 (DAD) detector at 224 nm was used for detection. The calibration curves obtained were linear (r^2=0.999) over the concentration range of 2-10 µg/ml, 0.4 – 2 µg/ml and 80-400 µg/ml for Empagliflozin, Linagliptin and Metformin respectively. The retention time of Empagliflozin, Linagliptin and Metformin was found to be about 6.1038min, 9.4754and 3.4846min respectively. The percentage (%) recovery was found within range for Empagliflozin, Linagliptin and Metformin hydrochloride.
As per the international Conference on Harmonisation (ICH, Q2) guideline, proposed RP–HPLC method validation has been carried out. The proposed RP–HPLC method was repeatable and selective as per statistical analysis and it can be used for simultaneous estimation of Empagliflozin, Linagliptin and Metformin hydrochloride.

Keywords: Liquid chromatography, development, validation, system suitability criteria, absolute difference, standard solution, sample solution, spiking, relative standard deviation.

ABBREVIATIONS:
ICH: International Council of Harmonization
RP–HPLC: Reverse phase high-performance liquid chromatography
DAD: Diode Array Detector
UV: Ultraviolet visible spectroscopy

[1]*,[2]Oriental University, Indore, Sanwer Road, Jakhya Opposite Revati Range Gate No.1, Indore, Madhya Pradesh 453555.[1]Email: kalpesh.raka@rediffmail.com , [2]Email: rohitkav10@gmail.com

***Corresponding Author:** Kalpesh Raka
*Department of Pharmacy, Oriental University, Indore, Sanwer Road, Jakhya Opposite Revati Range Gate No.1, Indore, Madhya Pradesh 453555. Telephone - +91 –9822069001, Email: kalpesh.raka@rediffmail.com

INTRODUCTION:

Metformin is an old and established first-line anti-diabetic medicine for the management of Type II diabetes mellitus.[1]The biguanide metformin (dimethyl biguanide) is an oral antihyperglycemic agent widely used in the management of non-insulin-dependent diabetes mellitus (NIDDM).[2] Metformin has an absolute oral bioavailability of 40 to 60%, and gastrointestinal absorption is apparently complete within 6 hours of ingestion. Metformin have Dissociation Constant (p^{Ka}) of 2.8, 11.5 (32°) and Log P*(octanol/water)* is − 2.6.It is soluble 1 in 2 of water and 1 in 100 of ethanol; practically insoluble in chloroform and ether.[3]The apparent volume of distribution (V/F) of metformin following single oral doses of 850 mg averaged 654 ± 358 L. Metformin is negligibly bound to plasma proteins. Metformin is excreted unchanged in the urine and does not undergo hepatic metabolism.[4]It is assumed that the pharmacokinetics of metformin absorption is non-linear.[5]

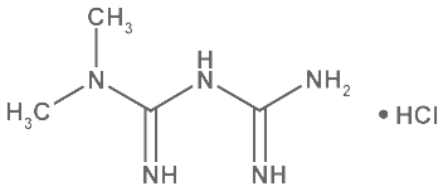


Figure 1: Structure of metformin hydro-chloride[6]

A white, hygroscopic, crystalline powder. Dissoci-ation constant (pKa) is 12.4.2.8, 11.5 (32°).[3] Partition coefficient (log $K_{o/w}$) is -2.64 at 25°C (est).Melting point is 232°C.[6]Ultraviolet spectrum in Methanol is at 236 nm (A^1_1=1163b).It is BCS class3.[7]

Empagliflozin is a medicine used to treat type 2 diabetes.[8] Empagliflozin is an orally available competitive inhibitor of sodium-glucose co-transporter 2 (SGLT2; SLC5A2) with antihyperg lycemic activity.[9]

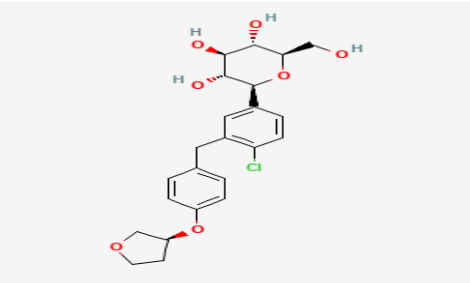


Figure 2: Structure of Empagliflozin[10]

Empagliflozin is indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus, to reduce the risk of cardiovascular death in adult patients with type 2 diabetes mellitus and established cardiovascular disease.[11]Empagliflozin is a white to yellowish, not hygroscopic solid powder. very slightly soluble in water (0.28 mg/mL), sparingly soluble in methanol (33.4 mg/mL), slightly soluble in ethanol (8.0 mg/mL), slightly soluble in acetonitrile (2.6 mg/mL), slightly soluble in 50% methanol in water (6.4 mg/mL), soluble in 50% acetonitrile in water (68 mg/mL), and practically insoluble in toluene (<0.001 mg/mL). Solubility data of empagliflozin in aqueous media at room temperature: Water (pH 8.6) 0.28 mg/mL; 0.1N HCl (pH 1.1) 0.30 mg/mL; McIlvaine buffer pH 4.0 (pH 4.1) 0.21 mg/mL; McIlvaine buffer pH 7.4 (pH 7.5) 0.14 mg/mL.[11]It is very slightly soluble in aqueous media between pH 1-7.5 but has low intestinal permeability (BCS class III).[12] Dissociation constant (p^{Ka}) strongest acidic (12.57) and strongest basic (-3). Partition coefficient (Log P) is 1.79, 1.66.[10]UV spectra against RO water as blank shows $\lambda_{max}$of 270.0 nm[13] and λ_{max}272nm in Methanol[14] and λ_{max}247nm in Ethanol.[15]

Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor which is used in combination with diet and exercise in the therapy of type 2 diabetes, either alone or in combination with other oral hypoglycemic agents. Linagliptin is a xanthine that is 7H-xanthine bearing (4-methylquinazolin-2-yl) methyl, methyl, but-2-yn-1-yl and 3-aminopiperid in-1-yl substituents at positions 1, 3, 7 and 8 respectively (the R-enantiomer).[16]

Linagliptin is an inhibitor of DPP-4, an enzyme that degrades the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulin tropic polypeptide (GIP). Thus, linagliptin increases the concentrations of active incretin hormones, stimulating the release of insulin in a glucose-dependent manner and decreasing the levels of glucagon in the circulation.[17]

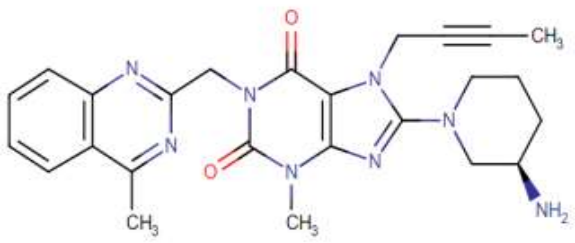


Figure 3: Structure of Linagliptin [16]

Linagliptin is white to yellowish, not or only slightly hygroscopic solid substance.[17] Slightly hygroscopic.[16] It is soluble in methanol (ca. 60 mg/mL), sparingly soluble in ethanol (ca. 10 mg/mL), very slightly soluble in isopropanol (< 1 mg/mL), and very slightly soluble in acetone (ca. 1 mg/mL).[17] It shows high solubility (> 1 mg/ml) in aqueous media up to pH 8.[18] It is very slightly soluble in water (0.9 mg/mL).[2] 0.0502 mg/mL.[19] Dissociation constant is p^{Ka1} is 1.9 and p^{Ka2} is 8.6. Partition coefficient (Log P) is LogP3-AA- 1.9[16], 2.62[19]. Melting point is190-196^{o}C.[19] BCS classification[4]is Class III (high solubility and low bioavailability).[18]

The aim of present study is to develop new, precise & simple assay content methodology by HPLC (High Performance Liquid Chromatography). To develop a method which determines simultaneous method in fixed dose combinations of solid oral dosage form. To decrease the time required for routine batch analysis which is performed by analyzing active separately. To develop a cost effective a method. To validate method for various parameters as per ICH Q2 (R1) guidelines.

MATERIAL AND METHODS:

Chemicals and reagents:

Metformin hydrochloride (100.7%), Empagliflozin (99.4%), Linagliptin (100.2%), Methanol, Ortho phospharic acid. All the drugs are used without further purification. All chemicals and reagents used were of HPLC grade and were purchased from reputed organization.

Apparatus:

Table 1: List of Instruments used for HPLC method development and validation

Sr. No	Name of Instrument	Use	Make/ Model
1.	HPLC	For HPLC analysis of sample	Agilet (1100)
	Software	For external control on HPLC instrument	Chemstation
2.	Cosmosil C18 (250 x 4.6 mm) 5 µ	For separation of component of mixture	Waters
3.	Electronic Weighing Balance	Weighing	Lab India
4.	Grade 'A' certified Glassware	For practical purpose	Borosil
5.	Ultrasonicator	For solubility & Degassing purpose	Biosystem
6.	pH meter	For pH adjustment of mobile phase	Lab India

Chromatographic Conditions

HPLC Agilent (1100) was used with software Chemstation. Column: 250 mm length x 4.6 mm internal diameter. Particle size packing: 5 µm. Stationary phase: C_{18} (COSMOSIL). Mobile phase: Methanol: 0.1 % OPA Water (30:70). Detection wavelength optimized as 224 nm. Flow: 0.7 ml/min. Temperature: Ambient, sample size: 20 µl.

Identification of Active Pharmaceutical Agents (API):

Following observations were performed to confirm identity of API.

Table 2: Identification of Metformin Hydrochloride

Sr. No.	Parameter	Results/observations		
		Metformin HCl	***Empagliflozin***	***Linagliptin***
01	Description	White crystalline powder	White powder	White powder
02	Solubility	Freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone and in methylene chloride.	Sparingly soluble in methanol, Slightly soluble in ethanol and Practically insoluble in water, Acetonitrile and toluene.	Soluble in methanol, slightly soluble in ethanol and practically insoluble in water.
03	Melting point	231-233°C	About 152°C	About 194°C

Solubility of API:

Based on literature survey; solubility studies were carried out to find out an ideal solvent in which all

three components are completely soluble. Various solvents as water, acetone, ethanol, methanol, methanol: 0.1 % OPA water (30:70) were tried for solubilisation of Metformin, Linagliptin and Empagliflozin in order to further finalize solvent and mobile phase.

From above study and from literature it is concluded that all three API are soluble in Methanol and 0.1 % OPA water. Hence Methanol and 0.1 % OPA water is selected as solvent for solubilisation and as mobile phase. The mobile phase and samples prepared for RP-HPLC analysis were filtered using 0.22 µm membrane filter (Millipore, Mumbai, India) and sonicated for 15 minutes for degassing before injecting into the HPLC system.

Selection or Detection of Wavelength

Stock solutions were prepared and UV spectrum were taken for all Metformin, Linagliptin and Empagliflozin. Spectrums are provided below.

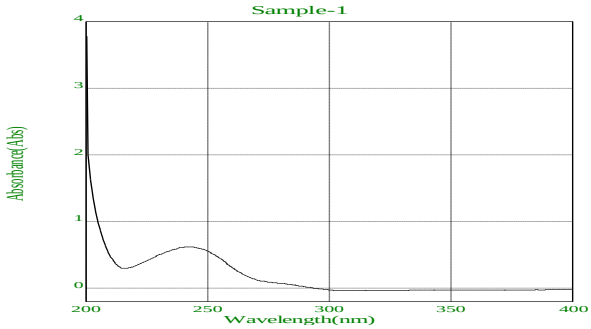


Figure 4: UV spectrum of Metformin in Methanol: 0.1 % OPA water (30:70)

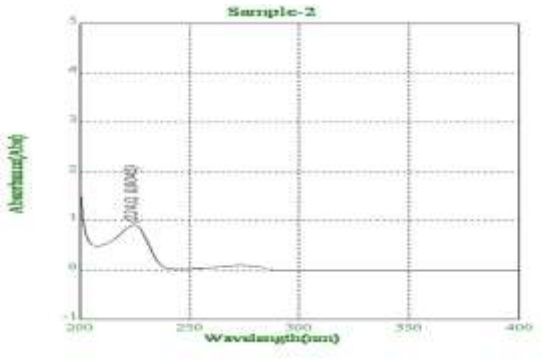


Figure 5: UV spectrum of Linagliptin Methanol: 0.1 % OPA water (30:70)

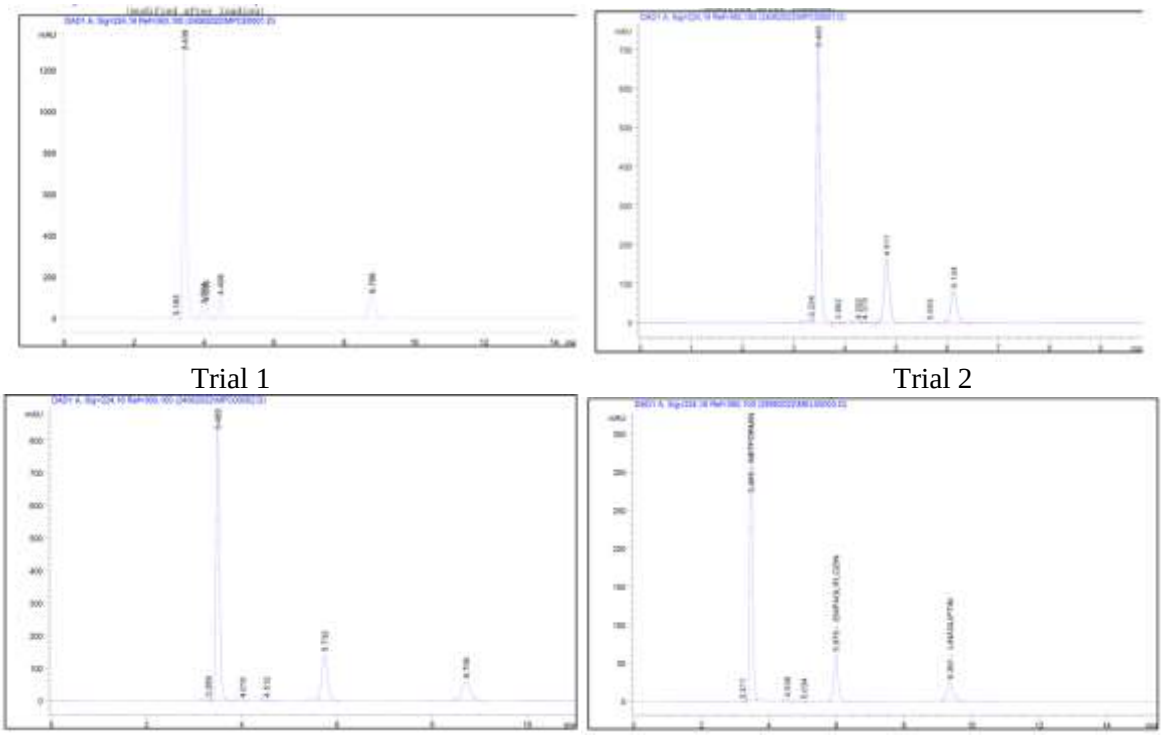
Figure 6: UV spectrum of Empagliflozin in Methanol: 0.1 % OPA water (30:70)

Analytical wavelength was selected from overlain UV- spectra of Metformin, Linagliptin and Empag liflozin. From the overlain spectra, it was observed that all the API showed good absorbance at 224 nm and therefore, this wavelength was selected as the analytical wavelength for detection.

Method Optimization:

The HPLC method was optimized with a view to develop assay method for simultaneous analysis of Metformin, Linagliptin and Empagliflozin. Aim of present study is to quantitate all API along with separation of impurities. Considering solubility of all API's and its characteristics RP-HPLC method seems to be suitable for separation and quantitation of these API's. Different combinations of mobile phases such as methanol: water in different combination and flow rate was evaluated in trials.

Initial trial (trial 1) was taken with water 30: methanol 70 as mobile phase, detection wavelength of 224 nm, flow 1.0 ml/min at ambient temperature and sample size: 20 µl. However resolution was not achieved and peaks were merged also tailing was observed. Hence next trial (trial 2) was conducted with change in composition of mobile phase to water 60: methanol 40. Still resolution and tailing was not achieved. Hence next trial (trial 3) was conducted with composition of mobile phase to water 30: methanol 70 and reducing flow rate from 1 ml/min to 0.7 ml/min. Resolution of Empagliflozin and Linagliptin is achieved. However resolution needs to be achieved for metformin peak. Next trial (trial 4) was carried out using mobile phase of 0.1 % OPA water 70: methanol 30and flow rate of 0.7 ml/min. Peaks of Metformin, Empagliflozin and Linagliptin were well resolved with no tailing. Also other impurity peaks were well resolved. Reproducibility was confirmed and method is further subjected to validation.



Trial 1 Trial 2

Trial 3 Trial 4

Figure 7: Representative chromatogram of trials

Method Validation:

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation

HPLC: Agilent (1100), ***Software:*** Chemstation, ***Column:*** 250 mm length x 4.6 mm internal diameter**,** ***Particle size packing:***5 µm, ***Stationary phase:***C_{18} (COSMOSIL), ***Mobile phase:*** Methanol: 0.1 % OPA Water (30:70), ***Detection wavelength:*** 224 nm, ***Flow:*** 0.7 ml/min, ***Temperature:*** Ambient, ***Sample size:***20 µl, ***Pump unit*** G1310A Iso-pump, ***Detector-*** G-13148 (DAD), ***Maximum Pressure*** **=** 400 Bar, ***Discharge Rate*** **=** 0.001 to 5 ml, ***Pressure limit range*** **=** 400 bar, ***Pressure display accuracy*** **=** 5 %, Pump unit HP-1100 Reciprocating pump

Preparation of Solutions:

i. ***Stock solution-1:*** Weigh accurately Metformin hydrochloride 200 mg, Empagliflozin 5 mg and 1 mg Linagliptin in 25 ml Methanol. (8000 µg/ml Metformin, 200 µg/ml Empagliflozin & 40 µg/ml Linagliptin.
ii. ***Solution-1*****:** Take 0.1 ml Stock solution-1 and make 10 ml with M.P = 80 + 2 + 0.4 µg/ml respectively.
iii. ***Solution-2:*** Take 0.2 ml Stock solution-1 and make 10 ml with M.P = 160+ 4 + 0.8 µg/ml respectively.
iv. ***Solution-3:*** Take 0.3 ml Stock solution-1 and make 10 ml with M.P = 240 + 6 + 1.2 µg/ml respectively.
v. ***Solution-4:*** Take 0.4 ml Stock solution-1 and make 10 ml with M.P = 320+ 8 + 1.6 µg/ml respectively.
vi. ***Solution-5:*** Take 0.5 ml Stock solution-1 and make 10 ml with M.P = 400+ 10 + 2.0 µg/ml respectively.

For Accuracy solution Preparation:-

Take 80+2+0.4 µg/ml solution for accuracy:
80 X 80 % = 64 µg/ml 2 X 80 % = 1.6 µg/ml 0.4 X 80 % = 0.32 µg/ml
80 X 100 % = 80µg/ml 2 X 100 % = 2.0 µg/ml 0.4 X 100 % = 0.4 µg/ml
80 X120 % = 96 µg/ml 2 X 120 % = 2.4 µg/ml 0.4 X 120 % = 0.48 µg/ml

1. System suitability:

Mixed standard solution was injected into the system and system suitability parameters were checked.

Table 3:System suitability

Sr. No.	Parameters	Acceptance limit	Results		
			Metformin	Empagliflozin	Linagliptin
1	Theoretical plates	Not less than 1500	8090	11467	13627

2	Tailing factor	Not less than 2.0	0.88	0.80	0.78
3	% RSD of Retention Time (Rt)	Not more than 2.0	0.17	0.14	0.32
4	% RSD of area	Not more than 2.0	0.88	0.61	0.43

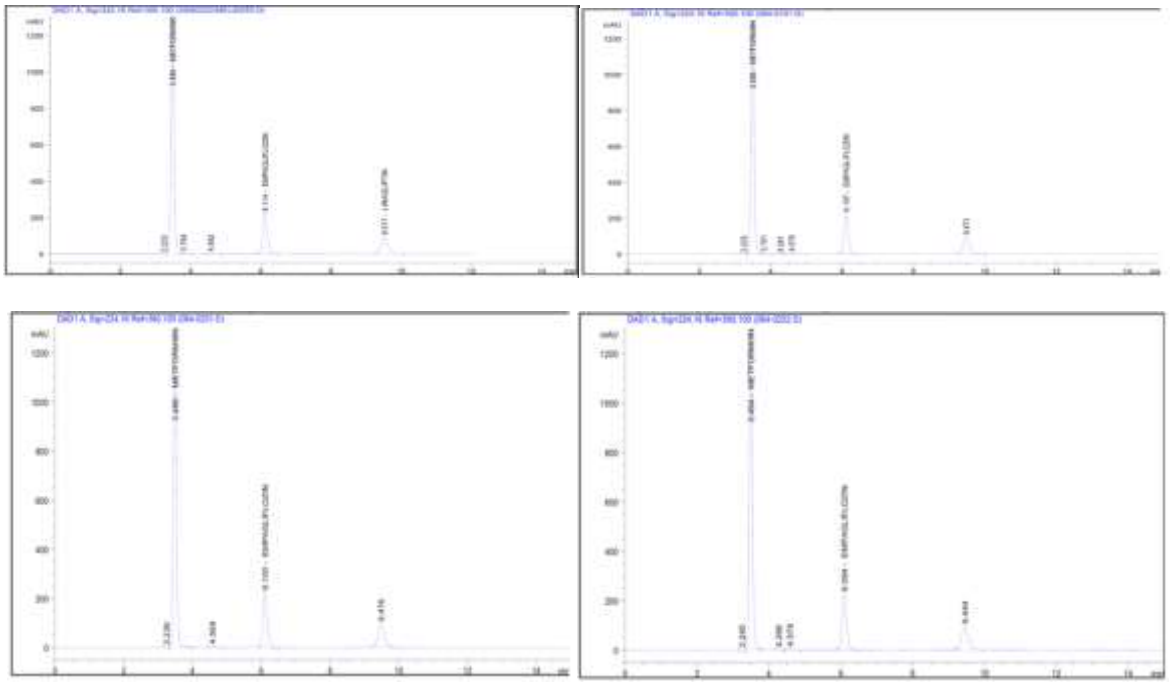


Figure 8: Representative chromatogram of system suitability

2. Linearity and Range:

Linearity solutions were prepared by stock solution of standard to obtain desired concentrations. A graph of corrected concentration (ppm) vs. area to beplotted. The correlation coefficient, Y-intercept deviation, % Y-intercept deviation, the slope of the regression line and residual sum of squares required to be reported.

Table 4:Linearity and Range of Metformin

Concentration	Area I	Area II	Area III	Mean	SD	%RSD
80	2198.5419	2200.346	2192.5467	2197.144833	4.082980114	0.19
160	4045.4626	4053.144	4125.2354	4074.614	44.00733552	1.08
240	5800.7788	5806.478	5914.3546	5840.536967	63.99141379	1.10
320	7672.2094	7709.178	7812.3784	7731.255167	72.64570215	0.93
400	9458.3808	9459.3085	9563.1232	9493.604167	60.20703575	0.63

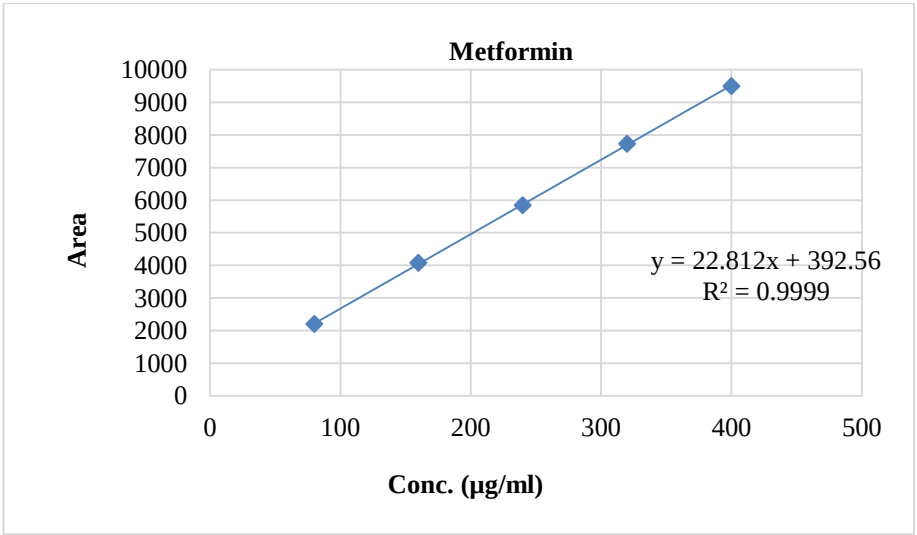


Figure 9:Linearity and Range of Metformin

Table 5:Linearity and Range of Empagliflozin

Conc.	Area I	Area II	Area III	Mean	SD	%RSD
2	522.4254	523.4254	524.9374	523.5960667	1.264666491	0.24
4	992.1625	991.3128	990.6475	991.3742667	0.759368068	0.08
6	1447.6470	1445.886	1446.3648	1446.632533	0.910613647	0.06
8	1939.2462	1918.435	1927.9352	1928.538833	10.41867317	0.54
10	2425.1169	2449.2773	2445.7463	2444.555933	5.415573239	0.22

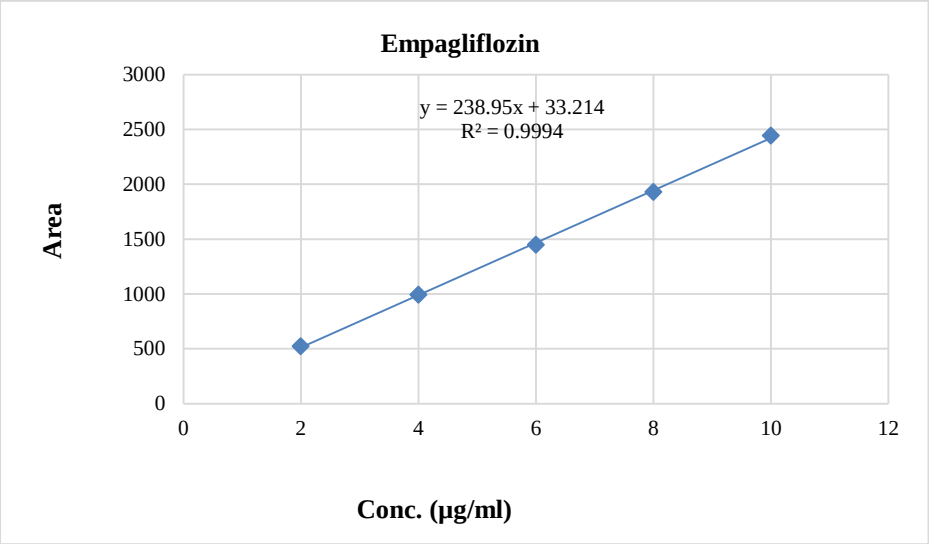


Figure 10:Linearity and Range of Empagliflozin

Table 6:Linearity and Range of Linagliptin

Conc.	Area I	Area II	Area III	Mean	SD	%RSD
0.4	305.0839	302.6079	302.2364	303.3094	1.54794727	0.51
0.8	594.0489	594.3229	590.4532	592.9416667	2.159425563	0.36
1.2	868.9245	869.6612	870.4219	869.6692	0.748732055	0.09
1.6	1174.3852	1168.351	1188.3102	1177.015467	10.23626608	0.87
2	1480.4090	1491.6957	1495.3608	1491.694333	3.637504436	0.24

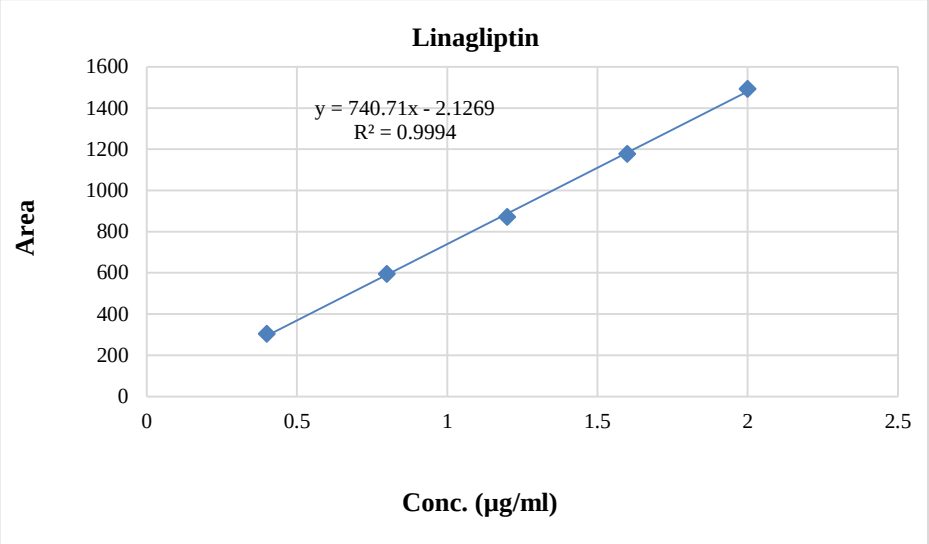


Figure 11:Linearity and Range of Linagliptin

Table 7: Result of linearity parameter:

Parameter	Metformin	Empagliflozin	Linagliptin
Linearity range (µg/ml)	80 - 400	2 - 10	0.4 - 2
Slope	22.812	238.95	740.71
Intercept	392.56	33.214	2.1269

Parameter	Metformin	Empagliflozin	Linagliptin
Correlation coefficient	0.9999	0.9994	0.9994

3. Precision:

Expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

a) Method precision

Single-injection of blank (diluent), six injections of different standard solution but same concentration and six preparation of sample solution were injected on the system. The % assay of Metformin, Empagliflozin and Linagliptin in each six samples was calculated. The results of method precision studies are shown in table.

Table 8: Results of method precision studies for Metformin

Sr. No	Conc. (µg/ml)	Area of STD	Area of sample	% Assay
1	240	5862.26	5787.46	98.72
2	240	5836.85	5846.97	100.17
3	240	5862.26	5764.23	98.33
4	240	5735.36	5845.36	101.91
5	240	5832.46	5799.76	99.44
6	240	5738.94	5801.78	101.09
Mean		5811.355	5807.59333	99.94333333
SD		58.8170491	32.7344734	1.384682876
% RSD		1.01	0.56	1.39

Table 9: Results of method precision studies for Empagliflozin

Sr. No	Conc. (µg/ml)	Area of STD	Area of sample	% Assay
1	6	1484.34	1475.83	99.43
2	6	1477.38	1476.43	99.94
3	6	1474.44	1474.48	100
4	6	1480.38	1477.57	99.81
5	6	1501.12	1499.33	99.88
6	6	1475.39	1476.53	100.08
Mean		1482.175	1480.02833	99.85666667
SD		9.95671382	9.50993253	0.228968702
% RSD		0.67	0.64	0.23

Table 10: Results of method precision studies for Linagliptin

Sr. No	Conc. (µg/ml)	Area of STD	Area of sample	% Assay
1	1.2	899.71	893.34	99.29
2	1.2	899.39	894.43	99.45
3	1.2	901.73	896.69	99.44
4	1.2	891.27	888.52	99.69
5	1.2	888.32	889.53	100.14
6	1.2	895.47	892.44	99.66
Mean		895.981667	892.491667	99.61166667
SD		5.28627058	3.05454034	0.2988924
% RSD		0.59	0.34	0.30

b) Repeatability

The analysis was carried out on three standard solutions by injecting triplicate lower, middle and upper concentration as described in methodology.

The mean, standard deviation and relative standard deviation of the result was calculated. The results of repeatability studies are shown in table.

Table 11: Results of repeatability studies for Metformin

Sr. No	Conc. (µg/ml)	Area I	Area II	Area III	Mean	SD	% RSD
1	80	2105.27	2105.23	2129.65	2113.3833	14.0873	0.67
2	240	5847.97	5838.56	5787.87	5824.8	32.3265	0.55
3	400	9316.97	9275.17	9297.86	9296.6666	20.9255	0.23

Table 12: Results of repeatability studies for Empagliflozin

Sr. No	Conc. (µg/ml)	Area I	Area II	Area III	Mean	SD	% RSD
1	2	525	527.85	526.75	526.5333	1.4373	0.27
2	6	1473.7	1474.57	1501.54	1483.27	15.8282	1.06
3	10	2438.37	2449.14	2398.54	2428.683	26.6545	1.09

Table 13: Results of repeatability studies for Linagliptin

Sr. No	Conc. (µg/ml)	Area I	Area II	Area III	Mean	SD	% RSD
1	0.4	302.8	304.41	304.96	304.0566	1.1225	0.37
2	1.2	896.87	899.33	897.87	898.0233	1.2371	0.14
3	2	1490.42	1490.78	1476.78	1485.9933	7.9810	0.54

c) Intermediate precision

Intermediate precision of the method was verified by different analyst carried out this analysis on a different day, using a different instruments and different column. The results of intermediate precision studies are shown in table.

Table 14: Results of Intermediate precision studies for Metformin

Sr. No	Conc. (µg/ml)	Analyst I (Day 1)	Analyst II (Day 2)	Analyst III (Day 3)	Mean	SD	% RSD
1	240	5862.26	5899.79	5917.12	5893.057	28.04297	0.48

Table 15: Results of Intermediate precision studies for Empagliflozin

Sr. No	Conc. (µg/ml)	Analyst I (Day 1)	Analyst II (Day 2)	Analyst III (Day 3)	Mean	SD	% RSD
1	6	1484.34	1487.31	1490.89	1487.513	3.279731	0.22

Table 16: Results of Intermediate precision studies for Linagliptin

Sr. No	Conc. (µg/ml)	Analyst I (Day 1)	Analyst II (Day 2)	Analyst III (Day 3)	Mean	SD	% RSD
1	1.2	899.71	906.06	908.30	904.69	4.455861	0.49

4. Accuracy

Recovery solutions were prepared by spiking stock solution of drug substance containing placebo to obtain solutions at 80 %, 100 % and 120 % of limit of target concentration.

Table 17: Accuracy at 80 % for Metformin

Sr no.	Amount added	Area	Amt received	% Received
1	64	3657.90	63.86	99.78
2	64	3647.4055	63.40	99.06
		Mean	63.63	99.42
		SD	0.327	0.51
		% RSD	0.514	0.51

Table 18: Accuracy at 100 % for Metformin

Sr no.	Amount added	Area	Amt received	% Received
1	80	4003.24560	79.07249	98.84
2	80	4008.89010	79.32115	99.15
		Mean	79.20	99.00
		SD	0.176	0.22
		% RSD	0.222	0.22

Table 19: Accuracy at 120 % for Metformin

Sr no.	Amount added	Area	Amt received	% Received
1	96	4460.619	99.22109	103.36

2	96	4447.7944	98.65614	102.77
		Mean	98.94	103.06
		SD	0.399	0.42
		% RSD	0.404	0.40

Table 20: Accuracy at 80 % for Empagliflozin

Sr no.	Amount added	Area	Amt received	% Received
1	1.6	896.46	1.59	99.12
2	1.6	893.1325	1.57	98.25
		Mean	1.58	98.68
		SD	0.010	0.62
		% RSD	0.628	0.63

Table 21: Accuracy at 100 % for Empagliflozin

Sr no.	Amount added	Area	Amt received	% Received
1	2.0	1001.85300	2.030238	101.51
2	2.0	1002.76890	2.034099	101.70
		Mean	2.03	101.61
		SD	0.003	0.14
		% RSD	0.134	0.13

Table 22: Accuracy at 120 % for Empagliflozin

Sr no.	Amount added	Area	Amt received	% Received
1	2.4	1086.726	2.388021	99.50
2	2.4	1123.9769	2.545051	106.04
		Mean	2.47	102.77
		SD	0.111	4.63
		% RSD	4.502	4.50

Table 23: Accuracy at 80 % for Linagliptin

Sr no.	Amount added	Area	Amt received	% Received
1	0.32	531.41	0.32	99.77
2	0.32	532.3062	0.32	100.15
		Mean	0.32	99.96
		SD	0.001	0.27
		% RSD	0.268	0.27

Table 24: Accuracy at 100 % for Linagliptin

Sr no.	Amount added	Area	Amt received	% Received
1	0.4	590.27800	0.399304	99.83
2	0.4	592.25210	0.401988	100.50
		Mean	0.40	100.16
		SD	0.002	0.47
		% RSD	0.474	0.47

Table 25: Accuracy at 120 % for Linagliptin

Sr no.	Amount added	Area	Amt received	% Received
1	0.48	651.031	0.481916	99.83
2	0.48	650.9459	0.4818	100.50
		Mean	0.40	0.48
		SD	0.002	0.0001
		% RSD	0.474	0.017

5. Robustness

Robustness of the method was verified by applying minor and deliberate changes in the experimental parameters, for eg. column temperature: ± 5 °C, flow rate: ± 0.2 mL/min, wavelength: ± 3 nm, mobile phase composition. Change was made to evaluate its effect on the method. Obtained data for each case was evaluated by calculating % RSD and % assay. The results of robustness studies are shown in table.

Table 26: Results of robustness studies for Metformin

Parameters	Area			Mean	SD	% RSD
	I	II	II			
Change in flow rate (ml)						
0.6	6027.19	6054.14	6040.53	6040.62	13.48	0.22
0.8	5960.86	6076.86	5992.32	6010.01	59.99	1.00
Change in mobile phase						
MeoH : Water (29 : 71)	5939.82	5962.4	5803.08	5901.77	70.39	1.19
MeoH : Water (31 : 69)	5939.44	5953.93	5992.31	5961.89	27.32	0.46
Change in wavelength (nm)						
223	5610.65	5659.54	5703.24	5657.81	46.32	0.82
225	5867.2	5915.33	5893.42	5891.98	24.10	0.41

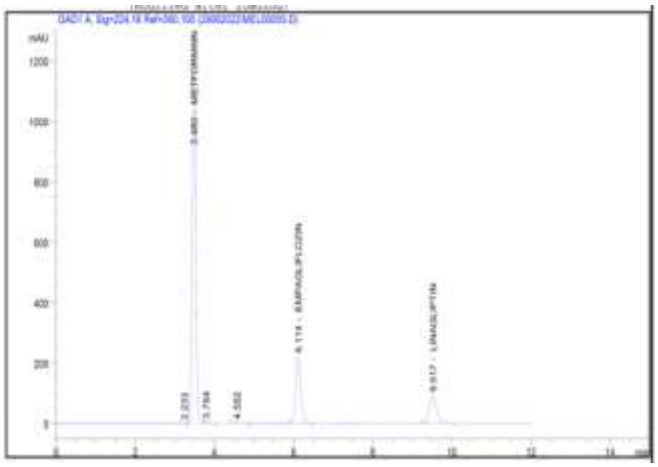
Table 27: Results of robustness studies for Empagliflozin

Parameters	Area			Mean	SD	% RSD
	I	II	II			
Change in flow rate (ml)						
0.6	1348.88	1346.14	1347.51	1347.51	1.37	0.10
0.8	1298.47	1302.13	1300.30	1300.3	1.83	0.14
Change in mobile phase						
MeoH : Water (29 : 71)	1505.08	1503.18	1504.1	1504.13	0.78	0.05
MeoH : Water (31 : 69)	1491.15	1493.73	1492.44	1492.44	1.29	0.09
Change in wavelength (nm)						
223	1459.29	1459.91	1459.6	1459.6	0.31	0.02
225	1524.33	1518.05	1521.19	1521.19	3.14	0.21

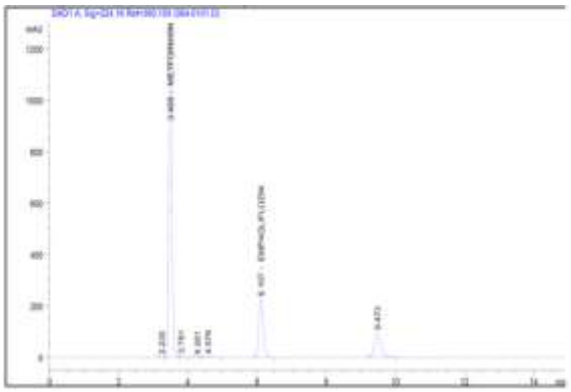
Table 28: Results of robustness studies for Linagliptin

Parameters	Area			Mean	SD	% RSD
	I	II	II			
Change in flow rate (ml)						
0.6	959.058	968.57	956.43	961.35	6.387	0.66
0.8	890.54	889.93	879.57	886.68	6.165	0.70
Change in mobile phase						
MeoH : Water (29 : 71)	912.07	910.82	913.45	912.11	1.074	0.18
MeoH : Water (31 : 69)	909.64	909.16	914.56	911.12	2.989	0.33
Change in wavelength (nm)						
223	992.49	968.6	957.45	972.84	17.901	1.84
225	906.71	911.29	910.89	909.63	2.537	0.28

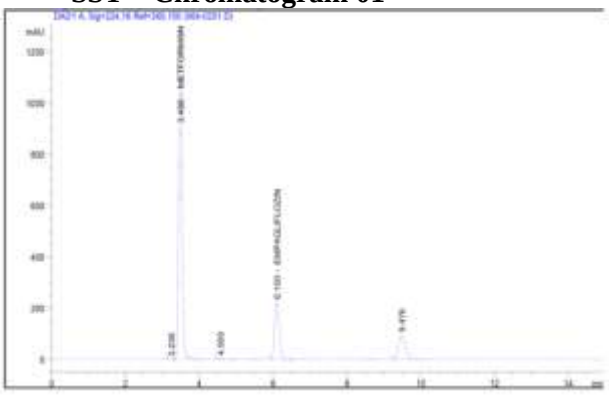
Blank



SST – Chromatogram 01

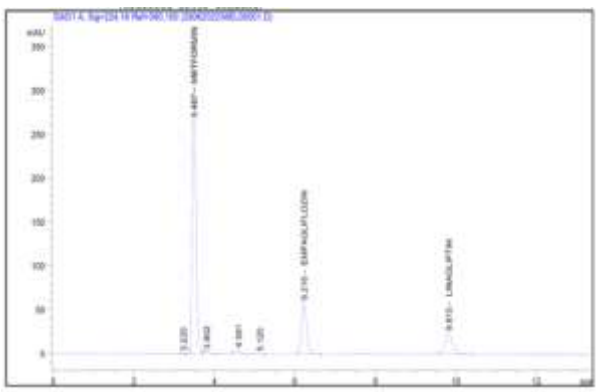


SST – Chromatogram 02

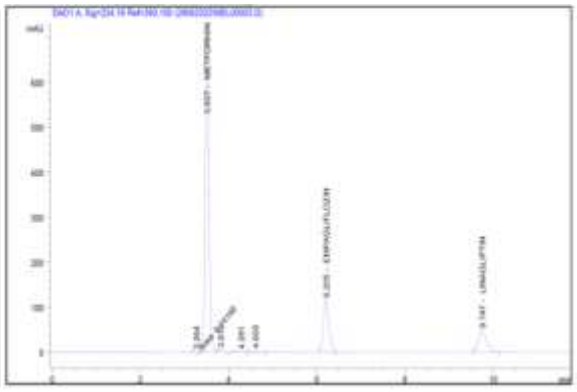


SST – Chromatogram 03

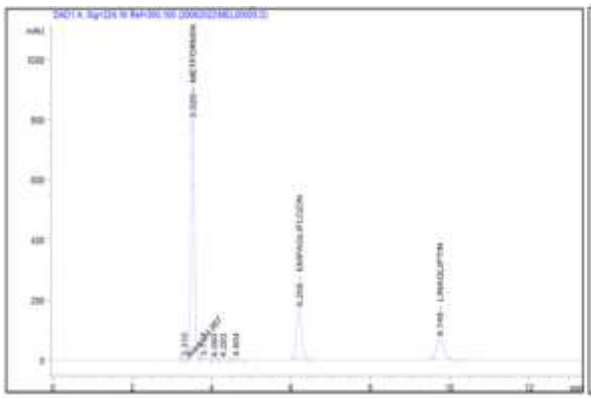
Figure 12:Representative chromatograms of system suitability



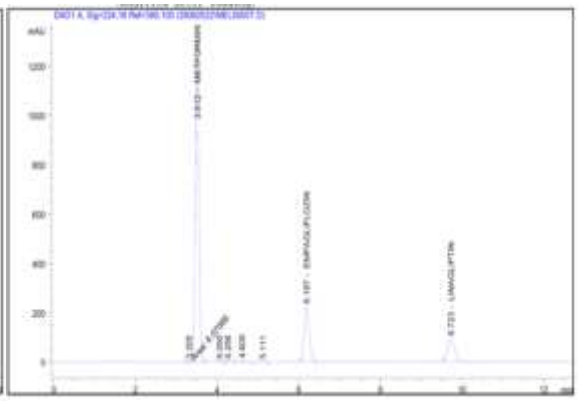
80+2+0.4



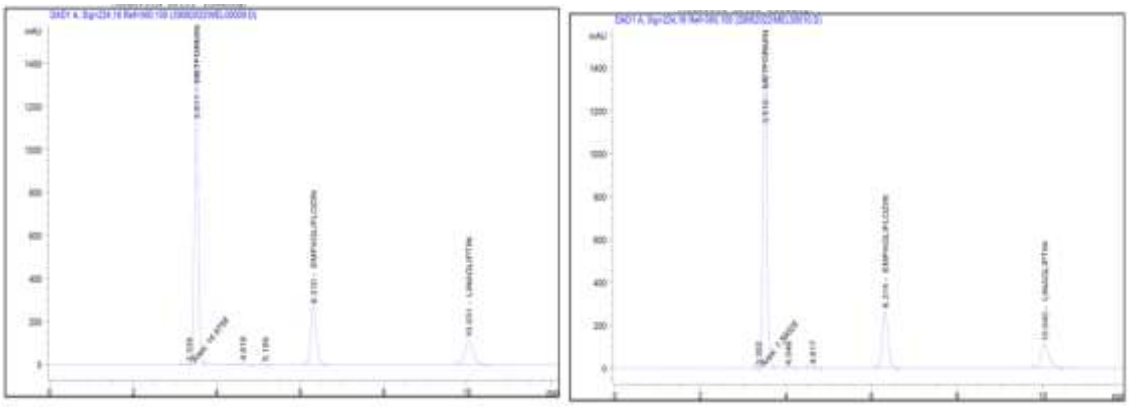
160+4+0.8 ppm



240 + 6 + 1.2 ppm

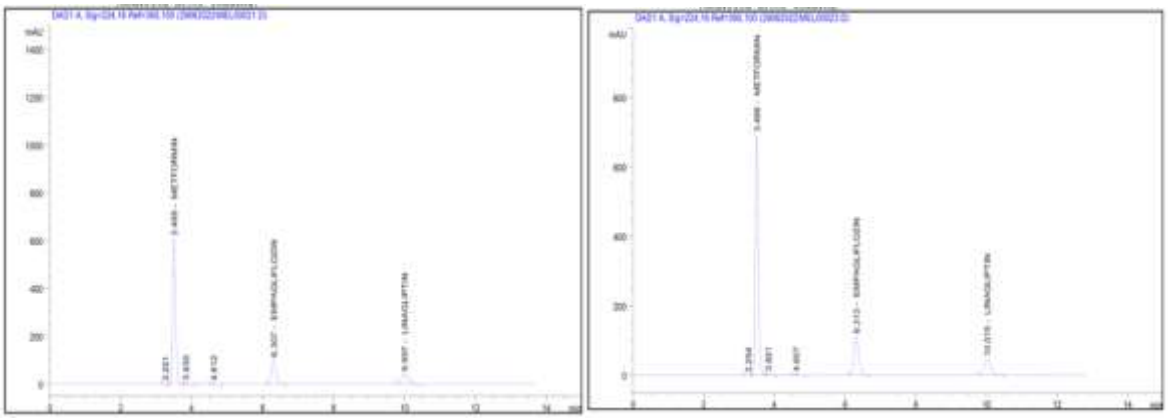


320+8+1.6-01 ppm

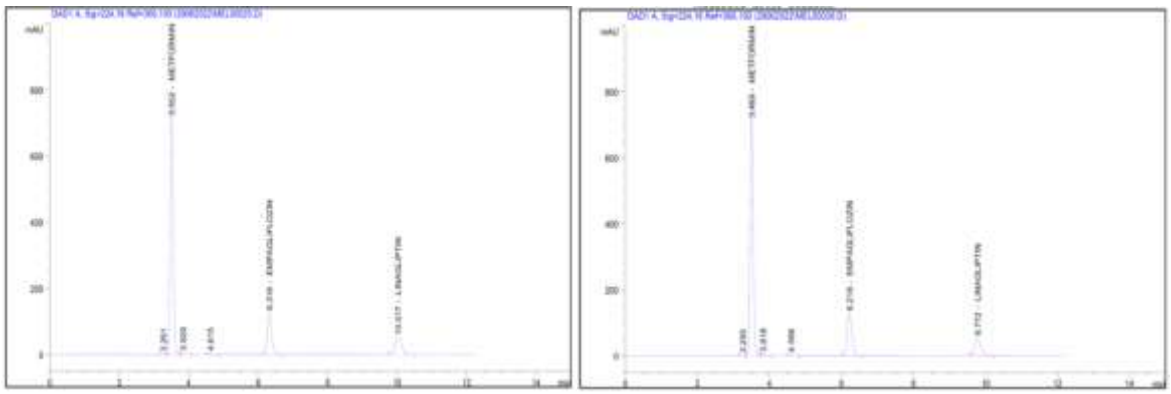


Linearity - 400+10+2 mcg – 01 **Linearity - 400+10+2 mcg – 02**

Figure 13:Representative chromatograms of Linearity and range



Accuracy (80 %) **Accuracy (100 %)**



Accuracy (120 %) – 01 **Accuracy (120 %) - 02**

Figure 14:Representative chromatograms of Accuracy

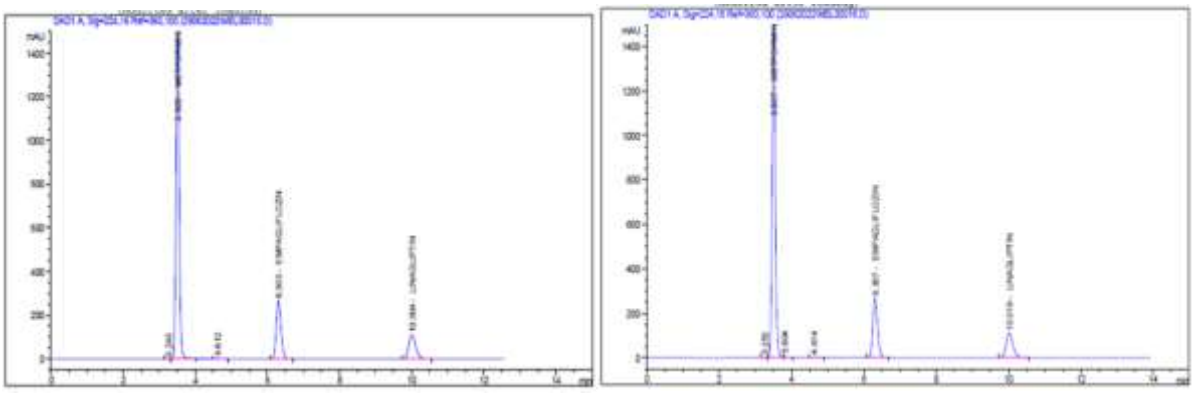


Figure 15:Representative chromatograms of Precision

DISCUSSION AND CONCLUSION

Analytical wavelength was selected from overlain UV- spectra of Metformin, Linagliptin and Empagliflozin. From the overlain spectra, it was observed that all the API showed good absorbance at 224 nm and therefore, this wavelength was selected as the analytical wavelength for detection. Based on literature and initial trials Methanol and 0.1 % OPA water was selected for mobile phase preparation. Flow rate was optimized to 0.7 ml/min.

The proposed HPLC method for estimation of the Metformin, Linagliptin and Empagliflozin simulta neously was applied successfully. Utilizing an isocratic mobile phase with commonly used column is very easy to perform to give acceptable and reproducible results. The validation of proposed method was carried out as per ICH guideline and performance data for all the parameters tested is acceptable. The method is found to belinear in the specified range, precise, and robust. Accuracy of the method is also established for the formulation. Hence, the proposed method is rapid, simple, and can be applied to quality control analyses of formulated product.

ACKNOWLEDGEMENT:

The authors are grateful to the authorities of Department of Pharmaceutical Analysis, Oriental University, Indore, India for providing opportunity to carry our research activities.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper

FUNDING SOURCE: None.

REFERENCES:

1. Australian Product Information of Galvumet® (2018). Vildagliptin/Metformin Hydrochlo ride Film-Coated Tablet. Novartis Pharma ceuticals Australia Pty Limited.
2. Electronic medicine compendia (2020). Eucreas® film-coated tablets. Novartis Pharma ceuticals UK Ltd.
3. Anthony C, David O. (2005). Clarke's analysis of drugs and poisons. Pharmaceutical press.3rd edition.
4. US-FDA prescribing information of Glucophage (Metformin hydrochloride) tablets". (2017). Bristol-Myers Squibb Company, USA.
5. European Medicine Compendia prescribing information "Glucophage film-coated tablets" (2019). Merck Serono Limited.
6. Access from: https://pubchem.ncbi.nlm.nih.gov/compound/Metfo rmin-hydrochloride
7. Annex 8, Proposal to waive in vivo bioequivalence requirements for WHO Model List of Essential Medicines immediate-release, solid oral dosage forms. WHO Technical Report Series, No. 937, 2006.Cited from: https://www.nhs.uk/medicines/empagliflozin/Cited from: https://pubchem.ncbi.nlm.nih.gov/compound/Empa gliflozin
8. Cited from: https://go.drugbank.com/drugs/DB09038
9. Canadian Product Monograph. PrSYNJARDY™ (Empagliflozin and metformin hydrochloride tablets). Boehringer Ingelheim (Canada) Ltd., April 2020.
10. Assessment report by EMA for Jardiance (empagliflozin). EMA/CHMP/137741/2014. Boehringer Ingelheim International GmbH, 20 March 2014.
11. Udai Bhan SR., Pradeep KG., "Novel UV Spectrophotometer Methods for Quantitative Estimation of Empagliflozin (EMPA) and Linagliptin (Lina) Using Mixed Hydrotropy Solubilization". Journal of Pharmaceutical Research International. 2021. 33(58A): 620-627.
12. Padmaja N., Mulagiri S., Development and validation of UV spectrophotometric method for Simultaneous estimation of Empagli flozin and Metformin hydrochloride in bulk drugs and combined dosage forms. Der Pharmacia Lettre, 2016, 8 (13), 207-213.
13. Jyothirmai N., Nagaraju B., Anil Kumar M., "Novel UV and Visible Spectrophotometric methods for the analysis of Empagliflozin a type 2 diabetic drug in bulk and pharmaceutical formulations". Journal de Afrikana. 2016, 3(1); 177-187.
14. Cited from: https://pubchem.ncbi.nlm.nih.gov/compound/Linagl iptin
15. US-FDA Prescribing information for Tradjenta™ (linagliptin) tablets of Boehringer Ingelheim Pharmaceuticals, Inc., 2011.
16. Chemistry Review(S) of Tradjenta (Linagliptin) Tablets (Application number: NDA 201280). Boehringer Ingelheim Pharmaceuticals, Inc. 2010.
17. Cited from: https://go.drugbank.com/drugs/DB08882