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Simultaneous Estimation method development and validation of Metformin, Empagliflozin and Linagliptin in Pharmaceutical dosage form by RP-HPLC

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ABSTRACT: This research includes the development of simple, rapid, precise and reproducible RP-HPLC for quality assurance of Metformin, Empagliflozin and linagliptin on Waters Alliance-e 2695 using X-Bridge phenyl column, mobile phase was Acetonitrile and potassium dihydrogen phosphate in ratio of (0/80, 5/80, 8/20, 10/80, 15/80) v/v. Flow rate was 1ml/min and wavelength of detection was 225nm through Photo diode array detector at ambient temperature. The number of theoretical plates and tailing factor were NLT 200 and should not be more than 2 respectively. %RSD of the peak areas of all measurements are greater than 2.

KEYWORDS: Empagliflozin, Linagliptin, Metformin, RP-HPLC

I. INTRODUCTION:

Chromatography tends to separate different compounds in a binary mixture using Mobile phase and stationary phase. It involves in passing the sample with analyte in the mobile phase often in the stream of solvent through stationary phase. Compounds passed through the stationary phase separates at different rates at specific time. Such that the time taken by each component to pass through is considered as Retention time. Chromatogram allows the chemical mixture of liquid or gas in order to separate into componential parts which results in differential distribution of solutes as they flow around stationary phase of solid or liquid. Separation of complex mixtures rely on differential affinities of substances for gas or liquid mobile medium and absorbing medium of stationary phase.

RP-HPLC: RP-HPLC method uses hydrophobic bonded packing materials usually Octyl or Octadecyl functional groups, polar mobile phase and often partial or fully aqueous mobile phase. Polar substances prefer mobile phase and elute first. As the hydrophobic nature of solutes increases, retention time increases. Lower the polarity of mobile phase, higher the elution strength.

PRINCIPLE: The main principle involving in the separation of binary mixture is by Adsorption and Partition. The sample of binary mixture gets injected, which enters into the column with the mobile phase. The components in the sample migrate through it passing between the stationary phase and mobile phase. This migration of particles was done due to the relative affinitive of the substances with stationary phase. Compounds move into column through mobile phase and migrate faster through column which compounds that tend to distribute in stationary phase migrate slower. Detector connected to outlet of column detects each compound eluting out from the column.

II. METHODS

Instrumentation: The chromatography separation was performed on Waters Alliance-e 2695 using X-Bridge phenyl column which is equipped with a quaternary pump with photodiode array detector.

Chemicals and reagents: Acetonitrile and potassium dihydrogen phosphate both were purchased from merk and water was purified by milli Q purification system. Metformin, Empagliflozin and Linagliptin were received from Hetero drugs pvt.Ltd.

Chromatographic conditions: The HPLC analysis was performed on Reverse phase chromatography by using X-Bridge phenyl column (150×4.6mm, 3.5µ). The mobile phase composition is Aceto-nitrile and potassium dihydrogen phosphate in ratios of (0/80, 5/80, 8/20, 10/80, 15/80) v/v. Flow rate was 1ml/min and wavelength of detection was 225nm through Photo diode array detector at ambient temperature. Mode of elution is Gradient.

DRUG PROFILE:

METFORMIN: Metformin is an anti-hyperglycemic agent of biguanides class used in

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the management of Type-2 DM. It has molecular mass of 165.62g/mol and Molecular formulae of $C_4H_{12}ClN_5$.It has an IUPAC name of 3-(di-amino-methylidene)-1,1-dimethylguanidine hydrochloride. Metformin was first approved in Canada 1972 and followed by 1995 in USA. Its melting point is 221ºC. Absolute bioavailability of 500mg Metformin was 50-60%. Steady state plasma concentration of Metformin is achieved within 24-48 hours.

EMPAGLIFLOZIN: Empagliflozin is an SGLT-2 inhibitor primarily responsible for reabsorption of glucose in kidney. It has molecular mass and molecular formula of 450.9g/mol and $C_{23}H_{27}ClO_7$ respectively. It has an IUPAC name of (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxy-phenyl] methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol. Half life was approximately 12.4 hrs and peak plasma concentration are achieved in 1.5 hrs. At steady state AUC and C_{max} were 1870nmol.h/L and 259nmol/L.

LINAGLIPTIN: Linagliptin is an anti-diabetic drug that works by increasing the levels of natural substances named incretins. It has Molecular mass and molecular formulae of 472.5 g/mol and $C_{25}H_{28}N_2O_2$. It has half life of 12 hrs. Peak plasma concentrations are 6-10nmol/L after single dose.

DETERMINATION OF WORKING WAVELENGTH:

The wavelength of maximum absorption of the solution of the drugs in mixture of Acetonitrile and KH_2PO_4 (0/80,5/80,8/20,10/80,15/80)were scanned using PDADetectorwithin the wavelength region of 200–400 nm against Acetonitrile and KH_2PO_4 (0/80,5/80,8/20,10/80,15/80)as blank. The absorption curve shows isobestic point at 225nm. Thus 225 nm was selected as detector wavelength for the HPLC chromatographic method.

PREPARATION OF STANDARD STOCK SOLUTION:

Accurately weigh and transfer 10mg of Metformin, 5mg of Empagliflozin and 5mg of Linagliptin into 10ml measuring flasks. Sonicate and make up to the marks with the solvent. Pipette out 0.5ml of Empagliflozin and 0.1ml of linagliptin solution to the Metformin solution and make up to the mark with diluent.

PREPARATION OF SAMPLE SOLUTION:

Accurately weigh and transfer 149.1mg of sample into a 10mL clean dry volumetric flask, add Diluent and sonicate it up to 30 mins to dissolve, and centrifuge for 30min to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45-micron Injection filter. (Stock solution). Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent

III. METHOD VALIDATION:

SPECIFICITY: Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose, blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drugs was specific.

SYSTEM SUITABILITY: Tailing factor for the peaks due to Metformin, Empagliflozin and Linagliptin in Standard solution should not be more than 2.0

Theoretical plates for the Metformin, Empagliflozin and Linagliptin peaks in Standard solution should not be less than 2000.

Resolution for the Metformin, Empagliflozin and Linagliptin peaks in standard solution should not be less than 2.

LINEARITY: 0.25ml, 0.5ml, 0.75ml, 1ml, 1.25ml, 1.5ml of the stock solutions were taken into individual volumetric flasks and were diluted with the diluent up to the mark. These concentrations of the solutions were loaded into the chromatographic system and plot area was measured.

RANGE: The Range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity

Acceptance Criteria:

Correlation coefficient should be not less than 0.999.

ACCURACY:

For preparation of 50% solution:

Accurately weigh and transfer 74.55 mg of sample into a 10 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution: Accurately weigh and transfer 149.1 mg of sample into a 10 ml clean dry volumetric flask add Diluent and sonicate

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to dissolve it completely and make volume up to the mark with the same solvent.Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution:

Accurately weigh and transfer 223.65 mg of sample into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102.0%

PRECISION: Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of 3 types

1. System precision
2. Method precision
3. Intermediate precision (a. Intraday precision, b. Inter day precision)

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analysed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyse the sample six times and calculate the % RSD.

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 1000ppm of Metformin, 25ppm of Empagliflozin and 5ppm of Linagliptin).

Acceptance Criteria:

The % RSD for the absorbance of six replicate injections results should not be more than 2%

ROBUSTNESS: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.8 ml/min to 1.2ml/min

Standard solution 30ppm 1000ppm of Metformin, 25ppm of Empagliflozin and 5ppm of Linagliptin was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±20%.

B. The variation of Organic Phase ratio

Standard solution of 1000ppm of Metformin, 25ppm of Empagliflozin and 5ppm of Linagliptin was prepared and analysed using the varied in mobile phase ratio

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

LOD for Metformin, was found to be 30µg/mL and LOQ for Metformin, was found to be 100 µg/ml, LOD for Empagliflozin was found to be 0.75 µg/ml and LOQ for Empagliflozin was found to be 2.5 µg/ml and LOD for Linagliptin, was found to be 0.15 µg/ml, LOQ for Linagliptin, was found to be 0.5 µg/ml.

DEGRADATION STUDIES

Acid degradation: Pipette 1 ml of stock solution into a 10ml volumetric flask and 1 ml of 1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials

ALKALI DEGRADATION: Pipette 1 ml of stock solution into a 10ml volumetric flask and add 1ml of 1N NaOH was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 1N HCl and make up to 10ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials

THERMAL DEGRADATION: Metformin, Empagliflozin and Linagliptin sample was taken in Petridis and kept in Hot air oven at 110^0 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

OXIDATIVE DEGRADATION: Pipette 1 ml above stock solution into a 10ml volumetric flask, 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min.

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Filter the solution with 0.45 microns syringe filters and place in vials.

HYDROLYTIC DEGRADATION: Pipette 1 ml above stock solution into a 10ml volumetric flask, 1 ml of water added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

PHOTOLYTIC DEGRADATION: Metformin, Empagliflozin and Linagliptin sample was taken in Petridis and placed under sunlight for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

REDUCTION DEGRADATION: Pipette 1ml of Stock solution transferred into 10ml volumetric flask to this add 1ml of 10% Sodium Bisulphate and kept on bench top for 10min then the remaining procedure is same as the test preparation.

IV. RESULTS AND DISCUSSION:

SYSTEM SUITABILITY:

Table 1: System suitability results of Metformin, Empagliflozin andlinagliptin were performed and listed below

S.no	Parameter	Metformin	Empagliflozin	Linagliptin
1	Retention time	2.969	4.022	5.010
2	Plate Count	3097	5095	6857
3	Tailing factor	1.15	1.12	1.06
4	Resolution	--	4.74	4.18
5	%RSD	0.65	0.47	0.61

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

LINEARITY:

Table 2: Linearity results of Metformin, Empagliflozin and Linagliptin were recorded below in the given table

S.NO	**Metformin**		**Empagliflozin**		**Linagliptin**	
	Conc.(µg/ml)	**Peak area**	**Conc.(µg/ml)**	**Peak area**	**Conc.(µg/ml)**	**Peak area**
1	250.00	861715	6.25	104301	1.25	59337
2	500.00	1463987	12.50	189653	2.50	132045
3	750.00	2097973	18.75	295299	3.75	190437
4	1000.00	2784341	25.00	378191	5.00	242224
5	1250.00	3587487	31.25	488539	6.25	305632
6	1500.00	4232107	37.50	570639	7.50	365632
Regression equation	y = 2781.17x + 60920.82		y = 15251.03x +3560.54		y = 48561.86x+2936.89	
Slope	2781.17		15251.03		48561.86	
Intercept	60920.82		3560.54		2936.89	

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R^2	0.9991	09995	0.9994

SYSTEM PRECISION:

Table 3: System precision results for Metformin, Empagliflozin and Linagliptin are given below

S. No	**Concentration Metformin (µg/ml)**	**Area of Metformin**	**Concentration of Empagliflozin (µg/ml)**	**Area of Empagliflozin**	**Concentration of Linagliptin (µg/ml)**	**Area of Linaglipti n**
1.	1000	2794621	25	375038	5	265354
2.	1000	2752861	25	374948	5	262874
3.	1000	2743349	25	373624	5	266034
4.	1000	2761143	25	376612	5	265341
5.	1000	2774786	25	371321	5	262218
6.	1000	2769302	25	374352	5	263021
Mean		2766010		374316		264140
S.D		17980.82		1768.76		1615.7
%RSD		0.65		0.47		0.61

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.65%, 0.47% and 0.61% respectively for Metformin, Empagliflozin and Linagliptin. As the limit of Precision was less than "2" the system precision was passed in this method.

REPEATABILITY:

Table 4: Method precision results for Metformin, Empagliflozin and Linagliptin are given below

S. No.	**Area for Metformin**	**Area for Empagliflozin**	**Area for Linagliptin**
1	2769274	375679	262319
2	2799553	372432	263247
3	2794189	373387	263054
4	2785510	377485	265138
5	2764115	372066	262055
6	2783067	371679	261359
Average	2782618	373788	262862
St.d	13781.66	2308.95	1309.23
%RSD	0.50	0.62	0.49

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INTERMEDIATE PRECISION:

Table 5: Intermediate precision results for Metformin, Empagliflozin and Linagliptin were discussed below

S. No.	Area for Metformin		Area for Empagliflozin		Area for Linagliptin	
	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
1	2791234	2786217	374786	373548	263430	261546
2	2791328	2779354	372367	371963	265320	264921
3	2791384	2790365	371542	372241	263461	263035
4	2723784	2742871	375143	374629	264312	265147
5	2773128	2765423	376357	375702	265761	266293
6	2713354	2721668	372687	372196	266124	265832
Average	2764035	2764316	373813	373379	264734	264462
Standard Deviation	36066.74	27071.42	1881.69	1528.85	1168.69	1813.77
%RSD	1.30	0.98	0.50	0.41	0.44	0.69

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

ACCURACY:

Table 6: Accuracy results of Metformin by RP-HPLC were given below

%Concentration(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1393139	50	50.37	100.7	100.5
100%	2794035	100	101.01	101.0	
150%	4145321	150	149.87	99.9	

Table 7: Accuracy results of Empagliflozin by RP-HPLC

%Concentration (atspecification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery
50%	187441	1.25	1.25	100
100%	375921	2.5	2.51	100.4
150%	562308	3.75	3.76	100.3

Table 8: Accuracy results of Linagliptin by RP-HPLC

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	132538	0.25	0.25	100	100.4
100%	263768	0.5	0.5	100	
150%	399137	0.75	0.76	101.3	

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Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.5%, 100.2% and 100.4% for Metformin, Empagliflozin and Linagliptin respectively.

Figure 1: Chromatogram of Accuracy 50%

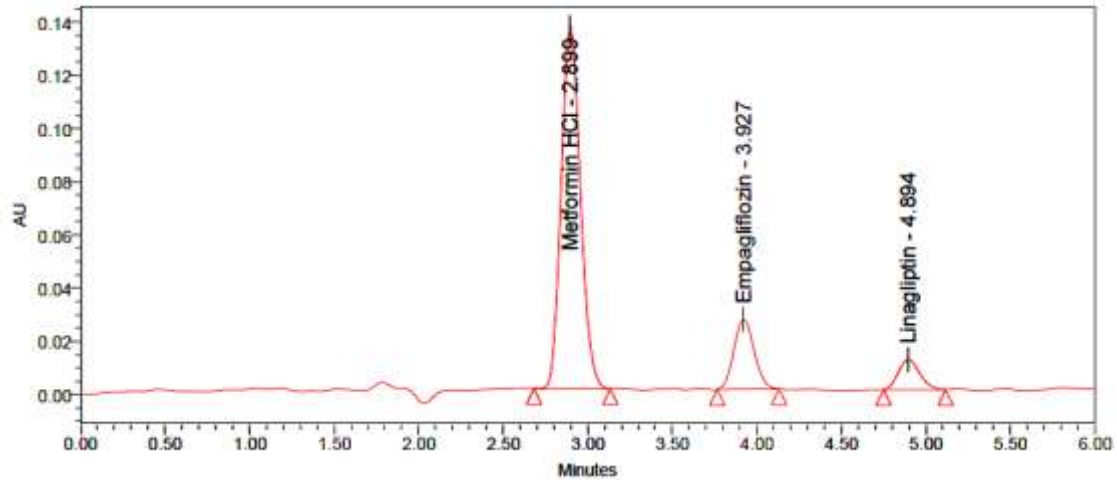


Figure 2: Chromatogram of Accuracy 100%

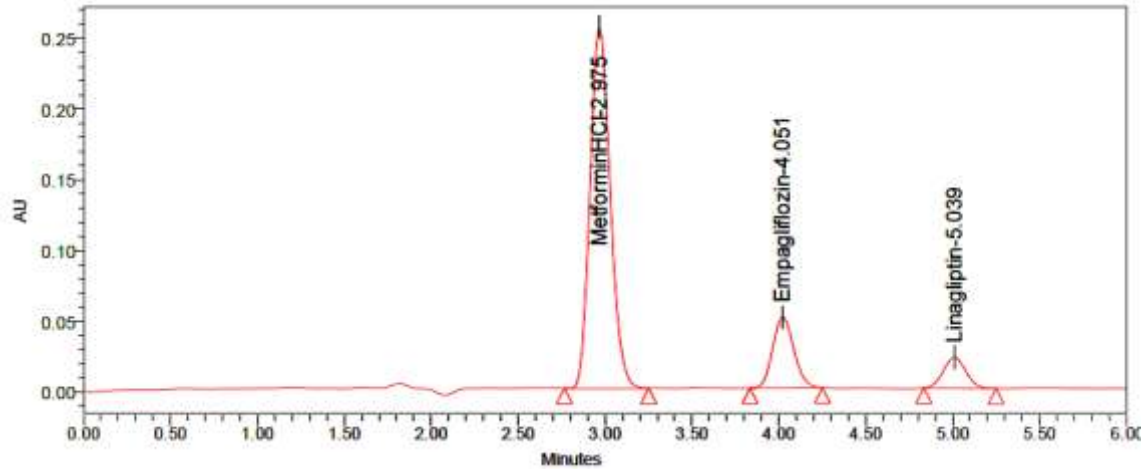
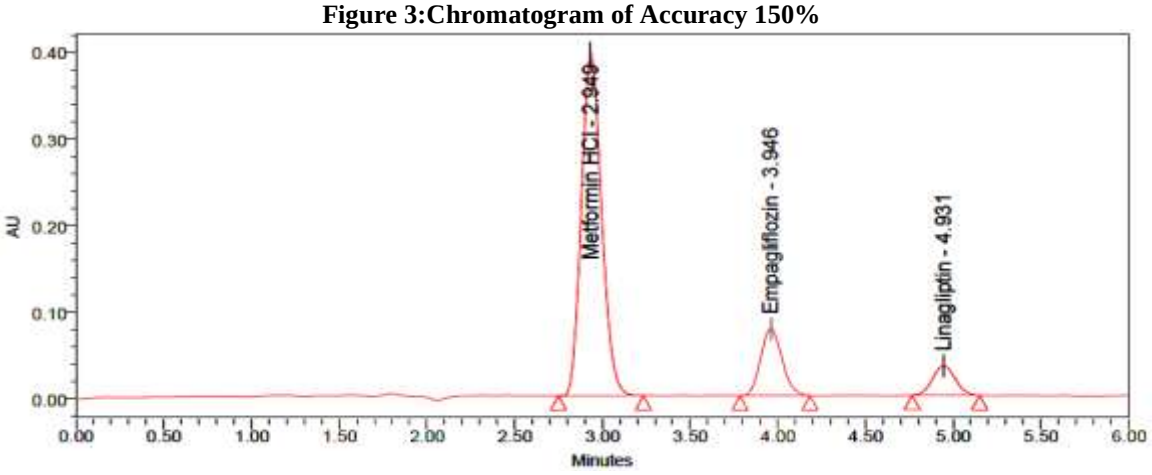


Figure 3:Chromatogram of Accuracy 150%



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ROBUSTNESS:

Table 9: Robustness results for Metformin by RP-HPLC

Parameter	Metformin					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change(mL/min)	Less flow(0.8ml)	3.257	3052641		1.15	3507
	Actual(1ml)	2.969	2794621		1.15	3097
	More flow(1.2ml)	2.673	2456982		1.14	3056
Organic Phase change	Less Org (0/72,5/72,8/18,10/72,15/72)	3.295	2994138		1.11	3123
	Actual(0/80,5/80,8/20,10/80,15/80)	2.965	2752861		1.11	3054
	More Org(0/88,5/88,8/22,10/88,15/88)	2.727	2494138		1.11	3225

Table 10: Robustness results for Empagliflozin by RP-HPLC

Parameter	Empagliflozin					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change(mL/min)	Less flow(0.8ml)	4.400	395623	4.85	1.03	5345
	Actual(1ml)	4.022	375038	4.74	1.12	5095
	More flow(1.2ml)	3.600	356298	4.21	1.11	5311
Organic Phase change	Less Org (0/72,5/72,8/18,10/72,15/72)	4.422	387542	4.60	1.16	5342
	Actual(0/80,5/80,8/20,10/80,15/80)	4.024	374948	4.51	1.18	5036
	More Org(0/88,5/88,8/22,10/88,15/88)	3.633	348502	4.59	1.03	5362

Table 11: Robustness results for Linagliptin by RP-HPLC

Parameter	Linagliptin					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change (mL/min)	Less flow(0.8ml)	5.483	304269	4.40	1.11	7001
	Actual(1ml)	5.101	265354	4.18	1.06	6857

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	More flow(1.2ml)	4.508	198524	3.89	1.04	7862
Organic Phase change	Less Org (0/72,5/72,8/18,10/72,15/72)	5.916	304268	5.25	1.04	7012
	Actual (0/80,5/80,8/20,10/80,15/80)	5.015	262874	4.26	1.02	6827
	More Org (0/88,5/88,8/22,10/88,15/88)	4.270	203257	2.33	1.17	7021

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

Table 12: Limit of detection and limit of quantification of Metformin, Empagliflozin and Linagliptin were recorded and listed below

Name of drug	LOD (µg/ml)	LOQ (µg/ml)
Metformin	30	100
Empagliflozin	0.75	2.5
Linagliptin	0.15	0.5

DEGRADATION STUDIES:

Table 13: Degradation studies for Metformin, Empagliflozin and Linagliptin byRP-HPLC

Results: % Degradation results	Metformin		Empagliflozin		Linagliptin	
	Area	% Degradation	Area	% Degradation	Area	% Degradation
Control	2759546	0.2	374065	0.1	264421	0
Acid	2356248	14.6	321643	14	230154	12.9
Alkali	2378955	13.8	318567	14.8	227543	13.9
Peroxide	2364514	14.3	316358	15.4	227845	13.8
Reduction	2335625	15.3	314851	15.8	226023	14.5
Thermal	2395218	13.2	322841	13.7	230634	12.8
Photolytic	2365715	14.2	336574	10	230340	12.9
Hydrolysis	2379823	13.7	331589	11.3	228754	13.5

V. CONCLUSION:

The developed RP-HPLC method was precise, rapid, easy and economical whereas the solvents used were inert and easy to prepare. The sample recoveries were in good agreement with their respective label claims and they suggested non-interference of formulation recipients in the estimation and can be used in laboratories for the routine analysis of selected drugs.The retention time for Metformin, Empagliflozin and Linagliptin were found to be 2.969, 4.022, 5.010. %RSD of Metformin, Empagliflozin and Linagliptin were 0.65, 0.47, 0.61.The percentage recoveries and retention times were good.

The present work concluded that stability indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the placebo and degradation products. Hence these can be used for routine analysis and Metformin, Empagliflozin and Linagliptin.

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