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ANALYTICAL METHOD VALIDATION REPORT FOR ASSAY OF METFORMIN HCL, EMPAGLIFLOZIN&LINAGLIPTIN BY RP-HPLC

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ABSTRACT
A New method was established for simultaneous estimation of Metformin, Empagliflozin, Linagliptin by RP-HPLC method. Chromatographic separations were carried using Thermo Hypersil, C18(4.6 x 150mm, 5µm) column with a mobile phase composition of 40% KH2PO4 buffer 60% Methanol have been delivered at a flow rate of 1ml/min and the detection was carried out using waters HPLC auto sampler, separation module 2695 with PDA detector 2996 at wavelength 224 nm.The retention time for Metformin, Empagliflozin, Linagliptin were 3.399, 5.586 and 6.439 minute respectively. The correlation coefficient values in linearity were found to be 0.999 and concentration range 600-1800 µg/ml for Metformin, 2-10 µg/ml for Empagliflozin and 1-5 µg/ml for Linagliptin respectively. For accuracy the total recovery was found to be 99.12 %, 100.48 % and 100.75 % for Metformin, Empagliflozin and Linagliptin respectively. The force degradation studies were performed for the dosage form and the results are within the limits. The results of study showed that the proposed RP-HPLC method is a simple, accurate, precise, rugged, robust, fast and reproducible, which may be useful for the routine estimation of Metformin, Empagliflozin, Linagliptin in pharmaceutical dosage form.

KEYWORDS: Metformin, Empagliflozin, Linagliptin, RP-HPLC, Simultaneous estimation.

INTRODUCTION
Metformin is an antihyperglycemic agent of the biguanide class, used for the management of type II diabetes. Metformin is considered an antihyperglycemic drug because it lowers blood glucose concentrations in type II diabetes without causing hypoglycemia. Metformin is commonly described as an *insulin sensitizer* leading to a decrease in insulin resistance and a clinically significant reduction of plasma fasting insulin levels.[1] Metformin's mechanisms of action are unique from other classes of oral antihyperglycemic drugs. Metformin decreases blood glucose levels by decreasing hepatic glucose production (gluconeogenesis), decreasing the intestinal absorption of glucose, and increasing insulin sensitivity by increasing peripheral glucose uptake and utilization. It is well established that metformin inhibits mitochondrial complex I activity, and it has since been generally postulated that its potent antidiabetic effects occur through this mechanism. The above processes lead to a decrease in blood glucose, managing type II diabetes and exerting positive effects on glycemic control.[2-3] IUPAC name 3-(diaminomethylidene)-1,1-dimethylguanidine. Molecular weight is 129.16. Molecular formula is $C_4H11N5.HCl$. It is freely soluble in water; slightly soluble in alcohol; practically insoluble in acetone and in methylene chloride.

Empagliflozin is an inhibitor of sodium-glucose co-transporter-2 (SGLT2), the transporters primarily responsible for the reabsorption of glucose in the kidney.[4] It is used clinically as an adjunct to diet and exercise, often in combination with other drug therapies, for the management of type 2 diabetes mellitus. The vast majority of glucose filtered through the glomerulus is reabsorbed within the proximal tubule, primarily via SGLT2 (sodium-glucose linked co-transporter-2) which is responsible for ~90% of the total glucose reabsorption within the kidneys. Na^+/K^+-ATPase on the basolateral membrane of proximal tubular cells utilize ATP to actively pump Na+ ions into the interstitium surrounding the tubule, establishing a Na^+ gradient within the tubular cell. IUPAC name 2-[4-chloro-3-[[4-[(3*S*)-oxolan-3-yl] oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol. Molecular Weight is 450.9. Molecular formula is C23H27ClO7. Empagliflozin is very slightly soluble in water (pH 1-7.4), slightly soluble in acetonitrile and ethanol, sparingly soluble in methanol, and practically insoluble in toluene.

Linagliptin is a DPP-4 inhibitor developed by Boehringer Ingelheim for the treatment of type II diabetes.[6] Linagliptin differs from other DPP-4 inhibitors in that it has a non-linear pharmacokinetic profile, is not primarily eliminated by the renal system, and obeys concentration dependant protein binding.[7]

Linagliptin is a competitive, reversible DPP-4 inhibitor. Inhibition of this enzyme slows the breakdown of GLP-1 and glucose-dependant insulinotropic polypeptide (GIP). GLP-1 and GIP stimulate the release of insulin from beta cells in the pancreas while inhibiting release of glucagon from pancreatic beta cells. These effects together reduce the breakdown of glycogen in the liver and increase insulin release in response to glucose. IUPAC name is 8-[(3*R*)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2-yl) methyl] purine-2,6-dione. Molecular Weight is 472.5. Molecular formula is C25H28N8O2. Linagliptin is soluble in methanol, sparingly soluble in ethanol, very slightly soluble in isopropanol, and very slightly soluble in acetone.

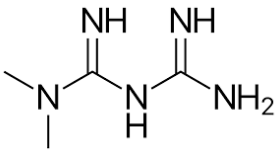

Fig no: 1 Structure of Metformin

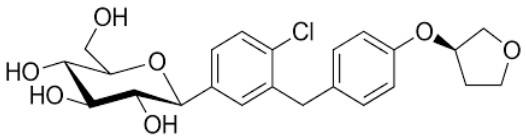

Fig no: 2 Structure of Empagliflozin

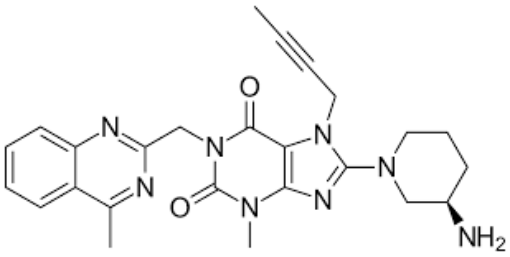

Fig no: 3 Structure of Linagliptin.

The literature survey revealed that There are very few methods reported in the literature for analysis of Metformin, Empagliflozin and Linagliptin alone or in combination with other drugs in the pure form and pharmaceuticals formulations. There was one UPLC[8] method reported for this three-drug combination where methanol: phosphate buffer pH 4 (50:50) employed as a mobile phase, on the other hand few analytical methods such as spectrophotometric[9–13], HPLC[14–18], UPLC[19] and LC–MS[20-21], which have been reported for analysis of these drugs alone or combination with other drugs. Hence, an attempt was made to develop RP-HPLC method for simultaneous estimation of metformin, empagliflozin and Linagliptin in pharmaceutical dosage form. It can be adopted in regular quality control test in industries and laboratories.

MATERIALS AND METHODS

Chemicals and Reagents: Metformin, Empagliflozin, Linagliptin were supplied by KP labs, Hyderabad. Dosage form was purchased by Local Pharmacy. Ortho phosphoric acid was analytical grade supplied by Finer chemical LTD, Mumbai, Water and Methanol for HPLC LICHROSOLV (MERCK)

Equipment and Chromatographic Conditions: The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, PDA detector and Empower 2 software. Analysis was carried out at 224 nm with an Thermo Hypersil, C18(4.6 x 150mm, 5µm) dimensions at ambient temperature. The optimized mobile phase consists of 40% KH2PO4 buffer 60% Methanol. Flow rate was maintained at 1 ml/min and run time for 10 min.

PREPARATION OF SOLUTIONS

Preparation of 0.1M KH2PO4 buffer

Accurately taken 2.68 gm in a 100 ml volumetric flask, dissolved and diluted to 100 ml with HPLC water and the volume was adjusted. The Ph of the solution was adjusted by using ortho phosphoroic acid.

Preparation of mobile phase

Accurately measured 400 ml (40%) of above buffer and 600 ml of Methanol were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

The diluents

The Mobile phase was used as the diluent.

Standard Solution Preparation

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin and 5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1000 ppm MET, 3 ppm of Lina and 6 ppm of EMPA)

Sample Solution Preparation

Accurately weigh 10 tablets of Each Drug and crush in mortor and pestle and transfer equivalent Tablet powder of Metformin, Empagliflozin and Linagliptin

individually to 1000 mg of Metformin and 5 mg Empagliflozin and 2.5 mg of Linagliptin samples into a 50mL clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of LINA and 6 ppm of EMPA)

Procedure

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Metformin, Empagliflozin and Linagliptin peaks and calculate the %Assay by using the formulae.

METHOD

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1ml/min for 10 minutes to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 10 µL of standard into Thermo Hypersil, C18(4.6 x 150mm, 5µm), the mobile phase of composition 40% KH2PO4 buffer 60% Methanol was allowed to flow through the column at a flow rate of 1ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Metformin, Empagliflozin and Linagliptin in their tablet dosage form. The result obtained for Metformin, Empagliflozin and Linagliptin was comparable with the corresponding labeled amounts and they were shown in Table-2.

Validation of Analytical method

Linearity and Range: Stock solution was prepared by dissolving the appropriate amount of Metformin, Empagliflozin and Linagliptin in 100 ml of diluent and further diluted to the required concentrations with diluent. The solution was prepared at five concentration levels ranging from 600 µg/ml to 1800 µg/ml of Metformin, 02 µg/ml to 10µg/ml of Empagliflozin, 1 µg/ml to 5 µg/ml of Linagliptin. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are shown in table 3.

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150%. Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Metformin, Empagliflozin and Linagliptin and calculate the individual recovery and mean recovery values. The results are shown in table 4-6.

Precision Studies: precision was calculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 7.

Ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 8.

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. The flow rate was varied at 0.9 ml/min to 1.1 ml/min. The results are shown in table 9-14.

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines.

Force degradation Studies

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Metformin, Empagliflozin and Linagliptin using the proposed method. The results are shown in table 15-17.

Preparation of stock

Accurately weigh and transfer 1200 mg of Metformin and 2.5 mg of Linagliptin and 5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Hydrolytic degradation under acidic condition

Pipette 0.3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.3 ml of above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the

volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal induced degradation

Metformin, Linagliptin and Empagliflozin sample was taken in petridish and kept in Hot air oven at 1100 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation

Pipette 0.3ml above stock solution 2 into a 10ml volumetric flask 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. Filter the solution with 0.45 microns syringe filters and place in vials.

RESULTS AND DISCUSSION

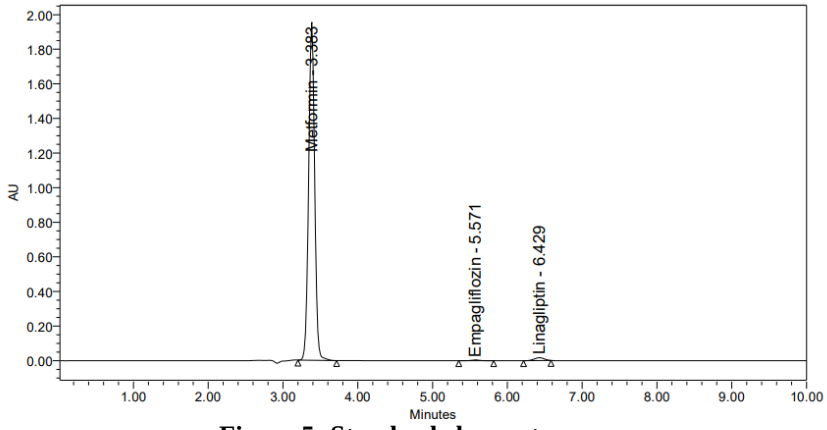

Figure 5: Standard chromatogram.

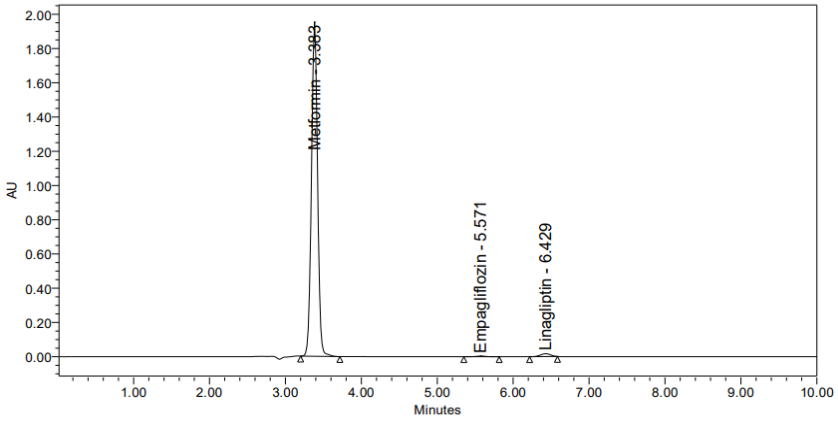

Figure 6: Sample chromatogram.

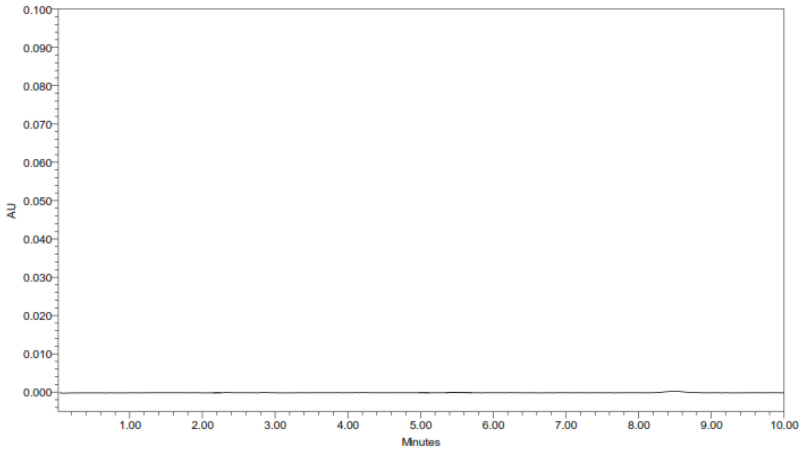

Figure 7: Blank chromatogram.

Table 1: System suitability parameters for Metformin, Empagliflozin, Linagliptin.

Parameters	Metformin	Empagliflozin	Linagliptin
Retention time	3.399	5.586	6.439
USP Plate count	8654	11451	9657
USP Tailing	1.00	1.07	1.02
USP Resolution	--	12.24	3.57

Table 2: Assay results for Metformin, Empagliflozin, Linagliptin.

	Label Claim (mg)	% Assay
Metformin	100	99.80
Empagliflozin	5	102.33
Linagliptin	2.5	99.80

Table 3: Linearity results for Metformin, Empagliflozin, Linagliptin.

S. No	Metformin		Empagliflozin		Linagliptin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	600	5533712	2	17070	1	87438
2	900	8409615	4	24837	2	134113
3	1200	11221992	6	33671	3	181266
4	1500	13768045	8	42017	4	230245
5	1800	16898907	10	51023	5	273289

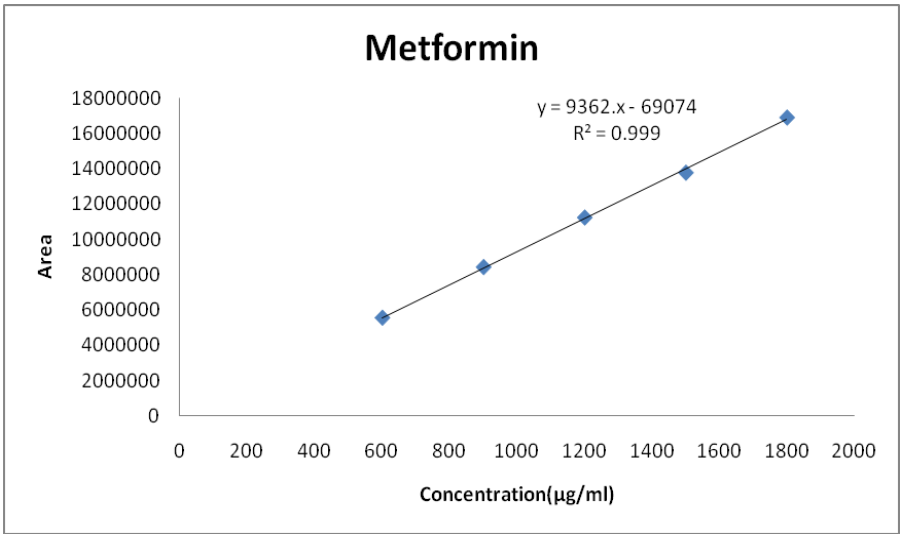

Figure 8: Linearity graph for Metformin

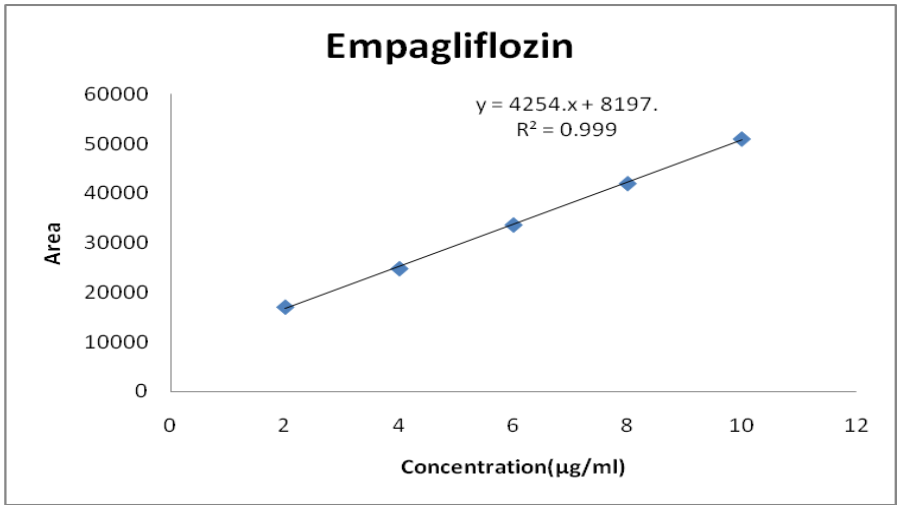

Figure 9: Linearity graph for Empagliflozin.

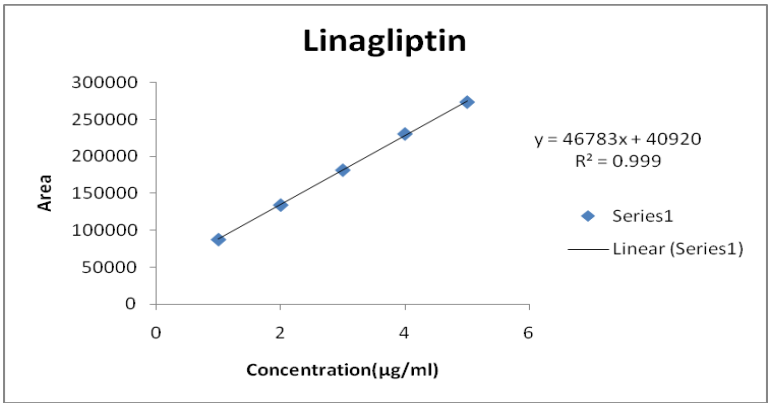

Figure 10: Linearity graph for Linagliptin.

Table 4: Accuracy results for Metformin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	5420274	500	496.44	99.29	99.12
100%	10905235	1000	998.80	99.88	
150%	16080832	1500	1472.8	98.19	

Table 5: Accuracy results for Empagliflozin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	16604	2.5	2.52	100.73	100.48
100%	33389	5	5.06	101.28	
150%	49166	7.5	7.46	99.42	

Table 6: Accuracy results for Linagliptin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	78504	1.25	1.26	100.94	100.75
100%	157002	2.5	2.52	100.94	
150%	234215	3.75	3.76	100.38	

Table 7: Precision results for Metformin, Empagliflozin, Linagliptin.

Injection	Area for Metformin	Area for Empagliflozin	Area for Linagliptin
Injection-1	10884334	32186	176519
Injection-2	10990504	32232	176202
Injection-3	11000201	32493	178059
Injection-4	10989075	32894	178530
Injection-5	10926237	33063	176142
Injection-6	10952941	33164	178382
Average	10957215.4	32671.8	177305.7
Standard Deviation	65743292.3	196031.0	1063834.2
%RSD	0.4	1.3	0.6

Table 8: Ruggedness results for Metformin, Empagliflozin, Linagliptin.

Injection	Area for Metformin	Area for Emapagliflozin	Area for Linagliptin
Injection-1	10884334	32186	176519
Injection-2	10990504	32232	176202
Injection-3	11000201	32493	178059
Injection-4	10989075	32894	178530
Injection-5	10926237	33063	176142
Injection-6	10952941	33164	178382
Average	10957215.4	32671.8	177305.7
Standard Deviation	65743292.3	196031.0	1063834.2
%RSD	0.4	1.3	0.6

Robustness Results

Table 9: Results for variation in flow for Metformin.

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	9160	1.03
2	1.0	8586.2	1.02
3	1.1	8548	1.02

Table 10: Results for variation in flow for Empagliflozin.

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	11774	1.02	12.38
2	1.0	11213.5	1.06	12.22
3	1.1	11089	0.98	11.96

Table 11: Results for variation in flow for Linagliptin.

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	10168	1.01	3.63
2	1.0	10847.2	0.96	3.71
3	1.1	9183	1.04	3.49

*Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 12: Results for variation in mobile phase composition for Metformin.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	9160	1.03
2	*Actual	8586.2	1.02
3	10% more	8548	1.02

Table 13: Results for variation in mobile phase composition for Empagliflozin.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	11774	1.08	12.38
2	*Actual	11213.5	1.06	12.22
3	10% more	11089	1.03	11.95

Table 14: Results for variation in mobile phase composition for Linagliptin.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	10168	1.01	3.63
2	*Actual	10847.2	0.96	3.71
3	10% more	9183	1.04	3.89

*****Results for actual Mobile phase composition have been considered from Accuracy standard.

Table 15: Degradation results for Metformin.

	Standard	Sample	% Assay	%Degradation
ACID	10896494	11443065	105.016	5.02
BASE	10896494	10619539	97.4583	-2.54
PEROXIDE	10896494	10292877	94.4605	-5.54
THERMAL	10896494	10242240	93.9957	-6
PHOTO	10896494	10206693	93.6695	-6.33

Table 16: Degradation results for Empagliflozin.

	Standard	Sample	% Assay	%Degradation
ACID	32901	34860	105.954	5.95
BASE	32901	30625	93.0823	-6.92
PEROXIDE	32901	31195	94.8148	-5.19
THERMAL	32901	30247	91.9334	-8.07
PHOTO	32901	30491	92.675	-7.33

Table 17: Degradation results for Linagliptin.

	Standard	Sample	% Assay	%Degradation
ACID	155236	160868	103.628	3.63
BASE	155236	141672	91.2623	-8.74
PEROXIDE	155236	172812	111.322	11.32
THERMAL	155236	144528	93.1021	-6.9
PHOTO	155236	145872	93.9679	-6.03

CONCLUSION

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Metformin, Empagliflozin and Linagliptin in pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Metformin, Empagliflozin and Linagliptin in pure and its pharmaceutical dosage forms.

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