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DEVELOPMENT AND VALIDATION METHOD FOR THE SIMULTANEOUS ESTIMATION OF EMPAGLIFLOZIN LINAGLIPTIN, AND METFORMIN IN SOLID DOSAGE FORMS BY OF RP-HPLC

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Abstract:

The purpose of the investigation was to develop a simple, rapid and accurate RP-HPLC method to determine assay of Linagliptin, Empagliflozin and Metformin in solid dosage forms. Simultaneous Estimation of Linagliptin, Empagliflozin and Metformin were carried out by RP- HPLC using trimethylamine buffer & acetonitrile and Agilent Eclipse XDB (250 mmx 4.6mm, 5µ) as a stationary phase and peak was observed at 240 nm which was selected as a wavelength for quantitative estimation. After the development of the method, it was validated for various parameters. It was found that recovery value of pure drug was between 99 % to 101% which indicates that the method is accurate and also reveals that commonly used excipients and additives present in the pharmaceutical formulations were not interfering in the proposed methods. The ruggedness of the method was checked by different analysts and found that the results were nearly same which indicates that the method is rugged. Based on the results observed, it was concluded that proposed method can be used for routine analysis of Linagliptin, Empagliflozin and Metformin.

Keywords: *RP-HPLC, Linagliptin, Empagliflozin and Metformin.*

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INTRODUCTION:

Empagliflozin is a sodium glucose cotransporter -2 (SGLT-2) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type-2 diabetes. SGLT2 cotransporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney.(2) The glucuretic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, therefore resulting in increased glucose excretion. Additionally, it contributes to reduced hyperglycaemia and also assists weight loss and blood pressure reduction. Linagliptin belongs to the class of Dipeptidylpeptidase 4-inhibitor,protease inhibitor.It is verysoluble in Ethanol, Water. Metformin Hydrochloride is a biguanide derivative which is the most commonly prescribed drug to treat hyperglycemia in individuals with Type 2 diabetes especially in overweight patients. Chemically it is known as1,1- dimethylbiguanide hydrochloride. Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.(1) These effects are mediated by the initial activation of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. The literature survey reveals that there are only few analytical methods available for estimation of Metformin Hydrochloride, Linagliptin and Empagliflozin individually and in combination are spectrophotometric and HPLC methods.(4-8) So we have planned to develop a simple, precise, economic and accurate Stability indicating RP-HPLC method development and validation for the estimation of Linagliptin, Empagliflozin and Metformin in solid dosage forms.

MATERIALS AND METHODS:

Active pharmaceutical ingredients Metformin Hydrochloride, Linagliptin and Empagliflozin were obtained as a gift sample from Hetero Drugs Limited, Hyderabad. The solvents used in this work were of HPLC grade and obtained from Rankem.

Preparation of buffer solution:

1ml of trimethylamine (TEA) was added to 1 L purified water in a 1000 ml volumetric flask, filtered, and sonicated to produce a 0.1 % TEA buffer solution. The pH of the resultant solution was adjusted to 3 by using a separately prepared and filtered orthophosphoric acid solution.

Preparation of mobile phase and diluent:

A mixture of 0.1%TEA (PH=3) and acetonitrile in a ratio of 40:60 (v/v) was prepared as a mobile phase and always sonicated before use. The same solvent solvent combination was utilized a diluent.

Preparation of standard solution

Weighed accurately, and transferred 5 mg of Linaglipitn, 25 mg of Empagliflozin, and 1000 mg of Metformin HCl, working standards into a 100 mL volumetric flask. Seventy milliliters of diluent was added to this mixture, sonicated for 15 minutes, and diluted to the mark to give a standard stock solution containing 0.05 mg/mL, 0.25 mg /mL, and 10 mg/mL of Linagliptin, Empagliflozin, and Metformin HCl respectively. Five milliliters of the stock solution was transferred into a 50 ml flask, and made up to the point with diluent to give a working standard solution having 5 µg/mL,25 µg/mL, and 1000 µg/mL of Linagliptin, Empagliflozin, and Metformin HCl respectively.

Preparation of sample solution:

Individually weighed 10 Trigardy XR® tablets labeled to contain 5 mg, 1000 mg, and 25 mg of Linagliptin, Metformin HCl, and Empagliflozin per tablet respectively,and calculated the average weight. Transferred the tablets into a mortar and finely powdered with a pestle. An amount of a powder equivalent to a weight of 1 tablet was transferred to a 100 mL volumetric flask. Seventy milliliters of diluent was added to this mixture, and sonicated for 15 minutes. The further volume was made up with diluent and then filtered with a 0.45 µ syringe filter. Transferred 5 mL from the above filtered stock solution into a 50 mL volumetric flask, and made up to the point with the diluent to yield a working sample solution having 5 µg/mL, 25 µg/mL, and 1000 µg/mL of Linagliptin, Empagliflozin, and Metformin HCl respectively.

Preparation of placebo solution:

Accurately weighed and transferred 1030 mg of placebo into a 100 mL volumetric flask; 70 mL of diluent was added, and sonicated for about 15 min. The further volume was made up with diluent and then filtered with a 0.45 µ syringe filter Transferred 5 ml from the above filtered placebo solution into a 50 mL volumetric flask, and made up to the mark with diluents

Preparation of calibration curve solutions:

An appropriate volume of aliquots from standard stock solution containing mixture of Linagliptin, Empagliflozin, and Metformin HCl (0.050 mg/mL, 0.25 mg /mL, and 10.0 mg/mL respectively) were transferred to seven different volumetric flasks. The resultant solutions were diluted with a diluent to

yield a calibration curve standard solution having a concentration of 0.5 - 7.5 µg/mL of Linagliptin, 2.5 - 37.5 µg/mL of Empagliflozin, and 100.0 - 1500.0 µg/mL of Metformin HCl.

Selection of detection wavelength:
Solutions of working standards for Metformin HCl, Linagliptin, and Empagliflozin were scanned in the wavelength regions of 200 – 400 nm by using the photodiode array detector. Good peak response was observed at 240 nm (isobestic point) for all drugs, and thus the same wavelength was selected as the detection wavelength for the study.

Method validation:
Method validation was performed following International Conference on Harmonization (ICH), Q2 (R1) guideline. The evaluated parameters were; linearity, accuracy, precision, robustness, the limit of detection, the limit of quantification, and specificity.

System suitability test:
The system suitability of the proposed RP-HPLC method was assessed to verify that the resolution and reproducibility of the system were adequate for the analysis to be performed. Six replicate determinations of freshly prepared working standard solution were performed by employing the optimized method to evaluate the system's suitability. Retention time (RT), Peak area (PA), number of theoretical plates (N), tailing factor (T), resolution (R), and %RSDs of the six determinations were computed and all the parameters were within the acceptable limit. The number of theoretical plates was >2000 in all chromatographic runs ensuring good column separation efficacy throughout the method development and validation process. In all cases Linagliptin, Empagliflozin, and Metformin HCl peaks were symmetric (tailing factor <2). The peaks were well resolved (R>2) and analytical outputs were reproducible (%RSD<2%). Thus, the system is suitable to perform the required analysis.

Table1: System suitability result of the proposed method

Peak	Statistic Parameter	Retention Time	Peak Area	Theoretical plates	Tailing Factor	Resolution
Metformin HCl	Mean	2.668	2793177	3042	1.07	-
	SD	0.006	3102.85	46.67	0.015	-
	%RSD	0.21	0.111	1.53	1.37	-
Linagliptin	Mean	3.588	263307	4530	1.06	3.42
	SD	0.004	312.76	60.79	0.015	0.06
	%RSD	0.124	0.119	1.34	1.38	1.76
Empagliflozin	Mean	5.413	378483	6902	1.11	5.78
	SD	0.007	314.38	58.90	0.016	0.10
	%RSD	0.12	0.083	0.85	1.49	1.73

Specificity:
Specificity was evaluated by performing chromatographic runs of blank, placebo solution, standard solution, and sample solution to cheek for interfering peaks (if any) in the chromatograms of blank and placebo. No interfering peaks appeared in the chromatograms of placebo and blank indicating that the formulation excipients didn't interfere with the determination of Empagliflozin, Linagliptin, and Metformin HCl simultaneously. Therefore, the proposed method was ascertained to be specific for the assay of the three drugs simultaneously. The representative specificity chromatograms are presented in Figures

Linearity:
Each of the seven calibration standard solutions was analyzed in triplicate, and calibration curves

were constructed by plotting the mean peak area against concentration. The resultant calibration curves were found to be linear for Linagliptin, Empagliflozin, and Metformin HCl in the investigated concentration ranges of 0.5-7.5 µg/mL, 2.5-37.5 µg/mL, and 100.0-1500.0 µg/mL respectively. The coefficient of determination (r^2) from least square regression analysis was ≥0.99 indicating the existence of a good correlation between peak area and concentration of analytes. Linearity result of the method is presented in Table 2.3, and calibration curves are depicted in Fig.

Table 2: Linearity data of proposed method

Metformin HCl		Linagliptin		Empagliflozin	
Concentration (µg/mL)	**Peak area**	**Concentration (µg/mL)**	**Peak area**	**Concentration (µg/mL)**	**Peak area**
100	299507	0.5	22738	2.5	39101
250	861715	1.25	59337	6.25	104301
500	1463987	2.50	122045	12.5	189653
650	1977973	3.75	190437	18.75	295299
1000	2794341	5.0	252224	25	378191
1250	3587487	6.25	301632	31.25	488539
1500	4232107	7.50	365632	37.5	570639
Regression equation $y = 2763x + 10115$ $r^2 = 0.998$		Regression equation $y = 49033x + 345.0$ $r^2 = 0.9993$		Regression equation $y = 15226x + 4176$ $r^2 = 0.9990$	

Precision:

The precision of the method was checked in two forms: repeatability and intermediate precision, and was expressed as %RSD. The repeatability was evaluated by performing six independent assays of the test sample solutions from the same homogeneous sample. The intermediate precision was evaluated by performing six independent assays of the same homogeneous sample on two consecutive days. The %RSDs of the method precision study was 0.51 for Metformin HCl, 0.57 for Empagliflozin, and 0.78 for Linagliptin. The % RSD values for the intermediate precision studies was 0.69 Metformin HCl, 0.79 for Empagliflozin, and 0.97% for Linagliptin. Results from both repeatability and intermediate precision evaluation were within the acceptance criteria of not more than 2.0 %, and thus the developed method was found to be precise. The results of the method and intermediate precision are presented in Table

Table 3: Intermediate precision result of the proposed method

Injection	Metformin HCl		Empagliflozin		Linagliptin	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
% Assay						
1	99.9	98.6	100.1	99.3	100.1	99.1
2	99.0	99.4	98.9	98.5	99.8	98.7
3	99.2	100.2	100.3	99.8	100.8	100.2
4	100.7	99.5	99.9	100.1	101.1	101.1
5	98.5	99.2	100.0	99.4	98.9	98.1
6	100.0	98.7	98.2	98.1	99.5	99.3
Mean	99.4		99.3		99.7	
SD	0.68		0.78		0.97	
% RSD	0.69		0.79		0.97	

a) Limit of detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ determinations were made to evaluate the sensitivity of the method, and it was assessed based on the signal-to-noise ratio (S/N). Analyte solutions at lower concentrations were prepared and analyzed in triplicates. LOD was identified as a concentration that yielded a S/N ratio of 3, and LOQ was identified as a concentration that yielded a S/N ratio of 10. LOD and LOQ result is presented in table 2.9. The lower LOD values witnesses the higher sensitivity of the method.

Table 4: LOD and LOQ result of the developed method

Parameter	Measured values (µg/ml)		
	Metformin HCl	Linagliptin	Empagliflozin
LOD	4.0	0.02	1.00
LOQ	13.4	0.07	3.3

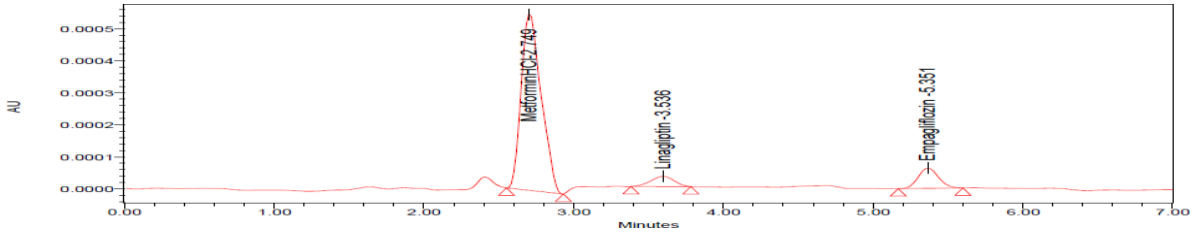

Fig. : LOD chromatogram of the proposed method

Robustness:

To evaluate the robustness of the method, working standard solutions were injected into the HPLC system at a flow rate variation conditions of 0.9 mL/min, and 1.1 mL/min, and a mobile phase composition variation conditions of 55: 45, v/v (acetonitrile: buffer), and 65:35, v/v (acetonitrile: buffer). All the system suitability parameters and %RSD values were computed. Good separation of the analyte peaks was achieved under all the robust conditions and the chromatographic parameter values remain largely unchanged. It can be seen from the results (Table 2.10) that no system suitability parameter was deviated from acceptable limit for all studied robust conditions. Tailing factors were <2, resolutions were > 2, plate counts were > 2000, and % RSDs of retention times were < 2. Thus, slight variations in the studied robust conditions didn't significantly affect analytical output, and this suggests that the proposed method was highly robust and reliable for routine analysis.

Table 5: Robustness data of proposed method

Optimized condition	Robust condition	Retention time	Theoretical plates	Tailing factor	Resolution	%RSD
Metformin HCl						
Flow rate (1 mL/min)	Minus (0.9 mL/min)	3.340	3937	1.13	-	0.77
	Plus (1.1 mL/min)	2.208	3292	1.11	-	0.53
Mobile phase composition (ACN: Buffer, 60:40 v/v)	Minus (55:45 v/v)	2.756	3112	0.99	-	0.78
	Plus (65:35 v/v)	2.658	3437	1.06	-	0.65

Linagliptin						
Flow rate (1 mL/min)	0.9	4.454	4549	1.13	4.85	0.46
	1.1	3.026	4451	1.05	3.33	1.05
Mobile phase composition (ACN: Buffer, 60:40 v/v)	55:45	3.822	4183	1.03	4.49	0.55
	65:35	3.451	4662	1.15	3.44	1.04
Empagliflozin						
Flow rate (1 mL/min)	0.9	6.671	6154	1.04	7.30	0.70
	1.1	4.446	6221	1.09	6.57	0.53
Mobile phase composition (ACN: Buffer, 60:40 v/v)	55:45	5.937	6493	1.05	7.59	0.78
	65:35	4.855	6531	1.07	6.66	0.65

Solution stability:

To evaluate the stability of sample and standard solutions, a freshly prepared solution was assayed at 0 hr (initially) and at 24 hr after leaving it on the bench top in a tightly capped flask at room temperature. The retention time and peak area of Metformin HCl, Linagliptin, and Empagliflozin remained almost similar and no significant degradation was found within the test period, and thus the solutions were stable at least for 24 hrs. Solution stability data are presented in Table .

Table 26: Solution stability result

Time	Metformin HCl		Linagliptin		Empagliflozin	
	% Assay	Difference	% Assay	Difference	% Assay	Difference
Initial	99.90	1.10	99.80	0.70	99.90	0.90
24 hr	98.80		99.10		99.0	

RESULT AND DISCUSSION:

The present analytical method was developed by studying different parameters. The ideal wavelength of PDA detection was selected at 240 nm because good peak response was obtained for all the three drugs at this wavelength. Better separation with good peak shapes was attained on Eclipse XDB C18 (250mm x 4.6mm, 5µ), and thus it was selected as a column for the proposed method. The mixture of 0.1% TEA (pH=3) and acetonitrile (40:60 v/v) was found to be the optimum mobile phase composition for the proposed study as it resulted in symmetric peaks with good resolution. The injection volume was selected to be 10 µL, and the flow rate was fixed at 1 mL/min for giving satisfactory retention times. Run time was selected to be 7 minutes because peaks were obtained around 2.660- 5.412 min for the three drugs.The developed method was validated according to ICH Q2R1 guidelines to apply the method for the analysis of the drugs in bulk and their formulations. The results of the validation parameters of the method lie within the prescribed limit. The system suitability results demonstrated that the method was reproducible and reliable for routine analysis, as all the parameters such as %RSD, theoretical plate count, tailing factor, and resolution were within the acceptance limit. The analytical method was found to be linear over the range 100.0-1500.0 µg/mL for Metformin HCl, 2.5-37.5 µg/mL for Empagliflozin, and 0.50-7.50 µg/mL for Linagliptin with a coefficient of determination (r^2) not less than 0.99 for the three analytes. The percent recovery was found between 98.0 and 102.0%, demonstrating the accuracy of the method. The method was precise because the % RSD values for repeatability and intermediate precision studies were less than 2%. The method was specific as no interfering peaks were observed from excipients. The method was robust, as small deliberate variations in method parameters such as flow rate, and mobile phase composition

didn't significantly influence analytical outputs.

CONCLUSION:

Stability-indicating RP-HPLC method has been effectively developed and validated for the assay of Empagliflozin, Linagliptin, and Metformin HCl simultaneously. The method was simple, fast, specific, cost-effective, and sensitive. Thus, it can be effectively used for quality control applications in pharmaceutical industries and drug control laboratories.

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