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RP HPLC- Reversephase liquid Chromatography, LOQ- Limit of Quantification, LOD- Limit of Detection, ICH- International conference of Harmonisation

Development and Validation of an RP-HPLC method for Simultaneous Estimation of Empagliflozin, Linagliptin and Metformin in bulk and Combined Pharmaceutical Dosage Forms

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ABSTRACT: Antidiabetic agents used for the treatment of type 2 diabetes mellitus, such as Empagliflozin, Linagliptin and Metformin are often used in combination therapy. The objective of this study was to develop and validate a simple, reliable and reproducible RP-HPLC method for simultaneous determination of Empagliflozin, Linagliptin and Metformin in tablet formulation. The separation by chromatography took place by using 0.1% glacial acetic acid and acetonitrile at the ratio of 55:45 (v/v) and detecting by wavelength 222 nm. Total analytical time was 12 min and the retention times were 1.967 min for Metformin, 4.477 min for Linagliptin and 7.40 min for Empagliflozin. The method exhibited good linearity over Metformin, Linagliptin and Empagliflozin concentration ranges from 300-800µg/mL, 1.5-4µg/mL and 3-8µg/mL respectively. R2 values were 0.998 for Metformin, 0.997 for Linagliptin and 0.994 for Empagliflozin. Percent recovery ranged from 99-102% and the %RSD was in the range of 0.01-0.06% for precision assessments. Empagliflozin, Linagliptin and Metformin had limits of detection and quantification of 0.375 µg/mL and 1.235 µg/mL, and 3.75 µg/mL and 12.3 µg/mL, respectively. Validation was conducted following the ICH guideline and the validation parameters investigated were satisfactory. The proposed method can provide reasonably good separation and reliable quantitation of all three drugs in a 12-minute run time and can be used for systematic QC testing of the drugs together in their pharmaceutical dosage forms.

INTRODUCTION

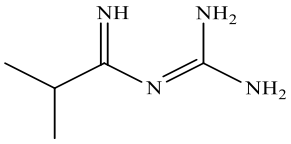
Diabetes Mellitus is an extremely prevalent disease in today's society. Metformin belongs to the class of antidiabetic medications known as biguanides; it is also an agent with antihyperglycemic activity. Lactic acidosis occurs very rarely with the use of metformin(1).

Linagliptin is an oral medicine that blocks the enzyme called dipeptidyl peptidase-4 (DPP-4). It acts by inhibiting an enzyme called dipeptidyl peptidase-4 (DPP-4) that is involved in the degradation of naturally occurring hormones that help regulate blood glucose (2).

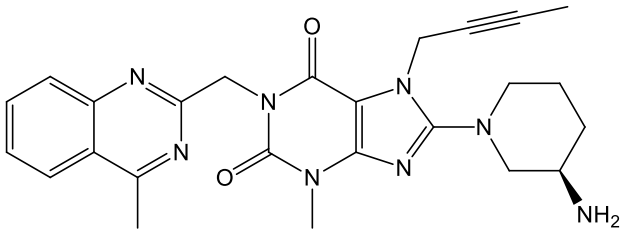
Empagliflozin belongs to a new class of drugs called SGLT2 inhibitors which inhibit sodium-glucose transport proteins filtered by the kidneys and reabsorbed back into the blood. Empagliflozin enables a substantial amount of sugar to be eliminated via urination(3).

Many dosage forms of various brands are available in the market, Empaglyn LM tablet with MET 1000mg, LINA 5mg, EMPA 10mg is used for Dosage forms study,There are very few Methods available for determination of Empa, Lina and Met by RP-HPLC method . An attempt was made to develop simple, sensitive and accurate method to determine and validate combined dosage forms.

1. Metformin



2. Linagliptin



3. Empagliflozin

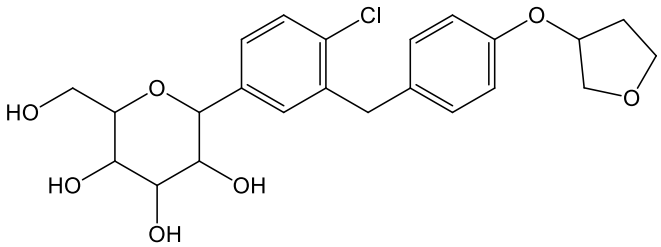


Fig 1: Chemical Structures of MET, LINA, EMPA

EXPERIMENTAL

Apparatus and Software:

The system used was an Agilent 1120 Compact LC HPLC system with a gradient pump (LC-10AT VP pump, 4 Mpa), Rheodyne injector and UV variable detector. Separations were done on a Waters XBridge C18 column (5 µm, 250 mm × 4.6

mm) under 222 nm UV detection. The dual-channel EZ Chrome Elite software (Acquisition and Evaluation) was used for the chromatographic data storage. An analytical balance (Shimadzu AUX 220) was used to weigh. Samples were sonicated using a sonicator (EQUITRON 230 V AC, 50 Hz). It was measured by a pH meter (Mettler Toledo compact S220, Germany). The use of HPLC-grade water (DQ3, Millipore, Germany) was used. The filtration throughout the experiment used a vacuum pump (SUPER FIT), a nylon membrane filter with a 0.45 µm pore size (Merck Millipore) and a filtration kit (TARSONS). It was done using a fin pipette (Thermo Scientific 1000 µl & 100 µl). For wavelength measurements, a double-beam UV–Visible spectrophotometer (SHIMADZU UV-1700) was used. These chemicals comprised glacial acetic acid (Merck AR grade), acetonitrile (Merck HPLC grade) and HPLC grade water.

Chromatographic conditions:

After several trials optimum conditions for separation of three drugs is given in table 1

Parameters	Result
Mobile phase optimized	GAA: Acetonitrile pH 3.2(55:45 v/v)
Stationary phase	Waters X Bridge C18 column (250 x 5 x4.6)
Flow rate	1 ml/min
Run time(RT	12 min
Temperature of the Column	25±1(ambient)°C
Volume of Injection	20 µl
Wavelength for detection	222nm

Table 1: chromatographic conditions for optimised method

Preparation of mobile phase:

0.1%Glacial Acetic acid, is prepared by usingWater of HPLC grade (20 mM). The pH of the solution was adjusted to 3.2± 0.1by using HPLC grade Sodium acetate. The study wascarried out using a binary pump, Channel A with HPLC grade GAA and Channel B with Acetonitrile.

Preparation of stock solutions:

The stock solution of metformin was prepared by weighing 100 mg of the drug and dissolving it in 100 ml of acetonitrile. The solution obtained was then used to prepare a stock solution with 1000µg/ml by taking 1ml of the solution and dissolving it in 100ml of acetonitrile. The stock solution of linagliptin was prepared by weighing 5mg and dissolving in 100 ml of acetonitrile and then 1ml of this was taken and dissolved in 10 ml of acetonitrile to give 5µg/ml. Stock solution of empagliflozin was prepared by weighing 5 mg of the drug and dissolving in 100 ml of acetonitrile, and subsequently 1 ml was transferred and dissolved in 10 ml acetonitrile, resulting concentration 5 µg/mL.

Preparation of working concentrations:

From stock solution3ml, 4ml,5ml, 6ml, 7ml,8ml of each drug of Metformin, Linagliptin and Empagliflozin and make up to 10ml with mobile phase(Glacial Acetic Acid and Acetonitrile55:45 ratio) in six 10 ml volumetric flasks. These 6 concentration are sonicated to remove water bubbles.

Chromatography Methodology:

Several trials were conducted with the various solvent combinations and ratios to choose the mobile phase that gives the following retention times (Rt): Metzanol 650: Acetonitrile 450 v/v (55:45 v/v; Rt = 1.967 min); linagliptin (Rt = 4.477 min); empagliflozin (Rt = 7.40 min). The wavelength was identified by scanning all three drugs over a wide range of wavelength from 200nm to 400nm. There was a reasonably good response at 222 nm.

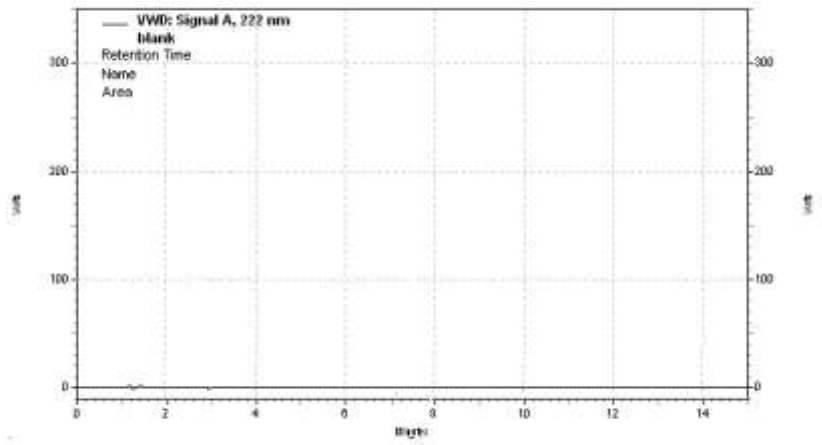


Fig 2: Chromatogram showing Blank run

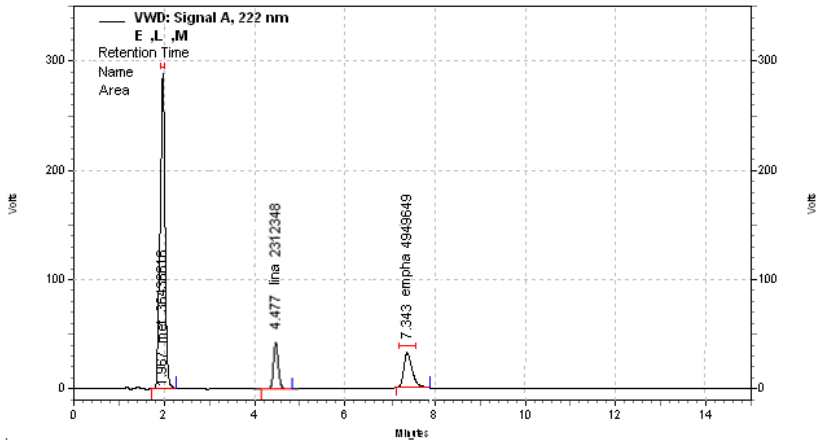


Fig 3: Chromatogram for optimised method

Linearity and Range:

A linear relationship between concentration and peak area is observed. 300- 800µg/ml of Metformin hydrochloride, 1.5-4 µg/ml of Linagliptin 3–8µg/ml of Empagliflozin, were prepared with 55:45 GAA: Acetonitrile(PH-3.2). Six serial dilutions were made for each of the above concentrations and each six serial dilutions 20µl of each concentration was injected into the HPLC system. They were then emitted and the chromatograms were recorded.

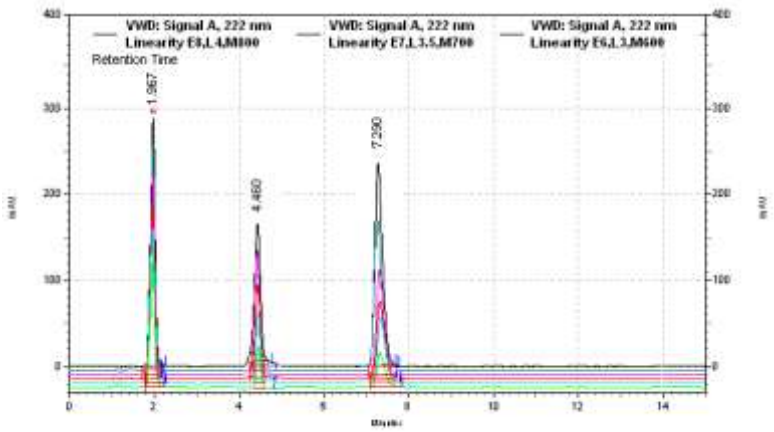


Fig 4: Chromatogram for linearity study

Precision:

The precision was determined in terms of intra-day (Morning and Afternoon) and inter-day (Day-1, Day-2) variation for a set (6 replicates) of drug solutions to check the response.

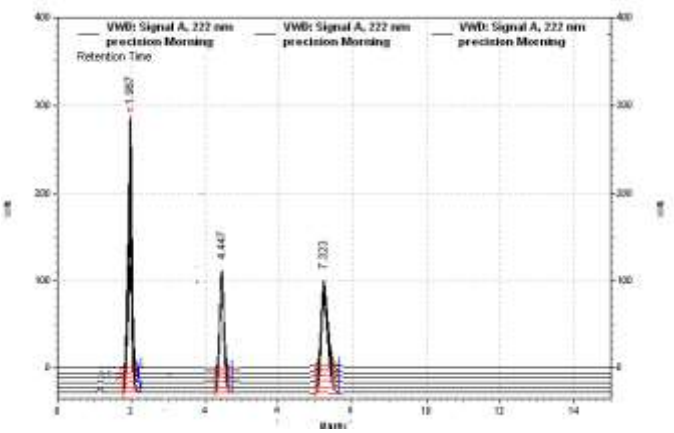


Fig 5: Chromatogram for Precision Morning result

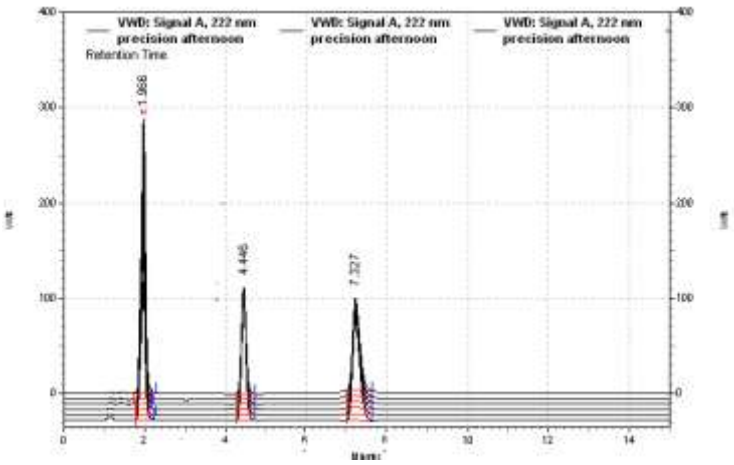


Fig 6: Chromatogram for Precision Afternoon result

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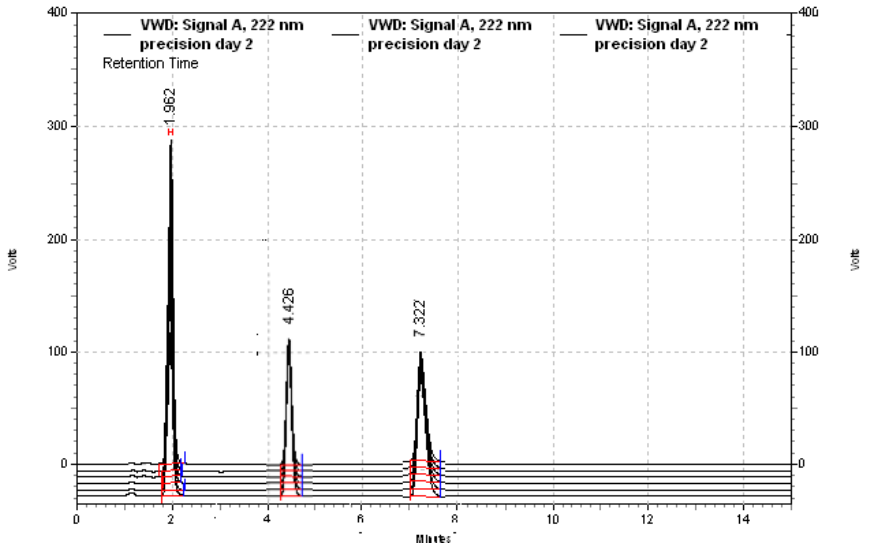


Fig 7: Chromatogram for Precision day 2 result

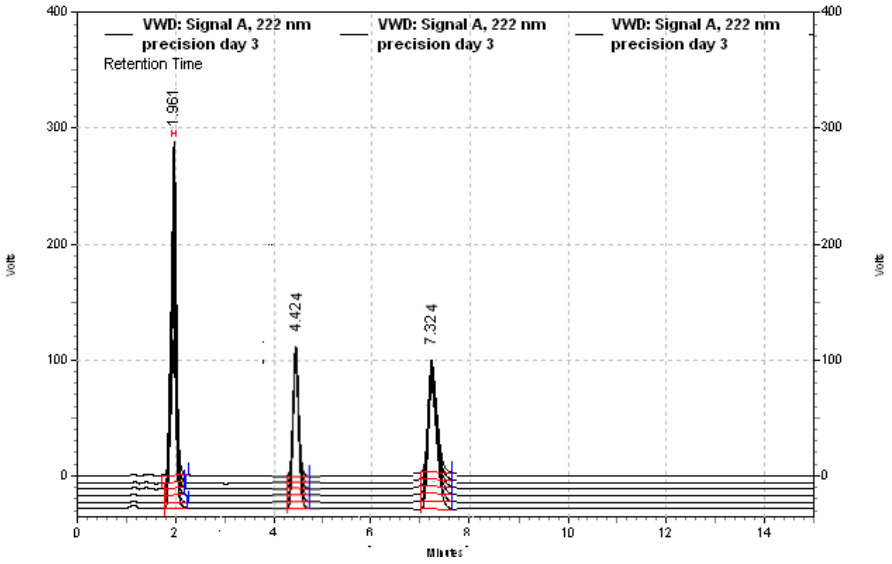


Fig 7: Chromatogram for Precision day3result

Accuracy

The accuracy was carried out for Metformin hydrochloride, Linagliptin and Empagliflozin at 80% (400µg/ml, 2µg/ml,4µg/ml), 100% (500µg/ml, 2.5µg/ml,5µg/ml)and 120% (600µg/ml, 3µg/ml,6µg/ml)levels and % recovery was determined.

Formulation used is EmpaglynLM manufactured by Pure and Care Health Care Pvt Ltd (Empagliflozin 10mg, Linagliptin 5mg, Metformin 1000 mg).

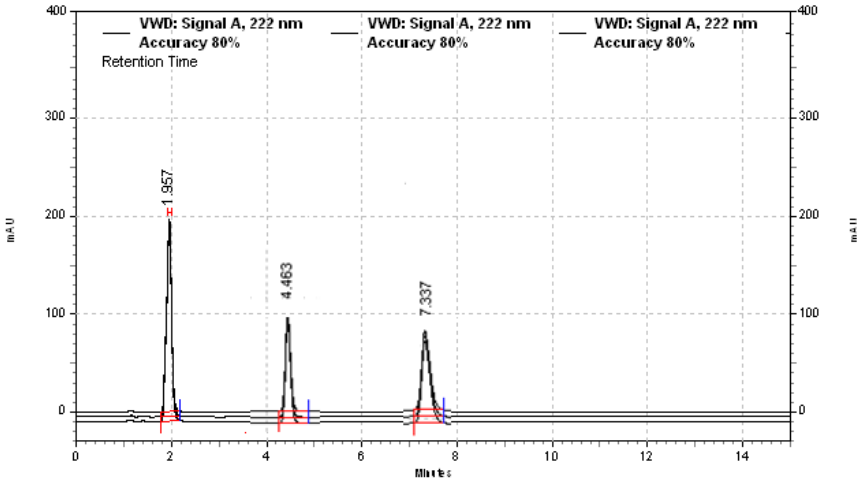


Fig 8: Chromatogram for Accuracy 80%result

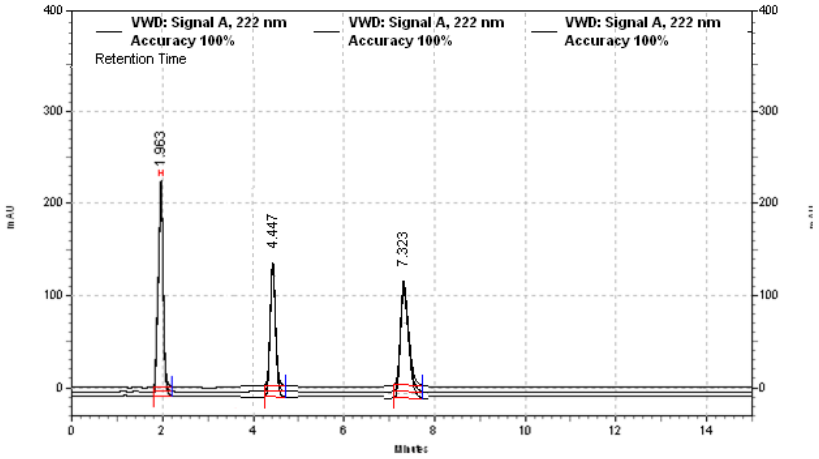


Fig 9: Chromatogram for Accuracy 100% result

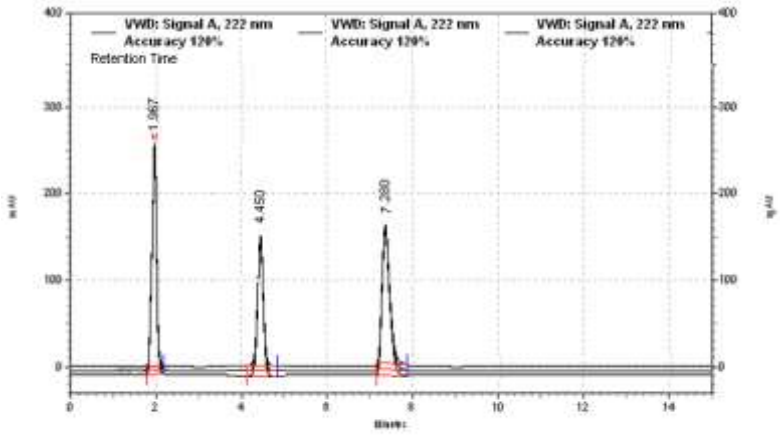


Fig 10: Chromatogram for Accuracy 120% result

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Robustness:

The strength of any procedure is its ability to not be affected by minor variations in the chromatography conditions according to ICH guidelines. Robustness is evaluated by changing the flow rate of the mobile phase by ±0.1 mL/min and the wavelength by ±1 nm from the wavelength maximum, and the system suitability is decided.

Limit of detection (LOD) and limit of quantification (LOQ):

The LOD and LOQ are determined according to ICH guidelines as a ratio of the signal to the noise. For calculating the LOD and LOQ, a signal-to-noise ratio of 3:1 and 10:1 is used, respectively.

RESULTS AND DISCUSSION

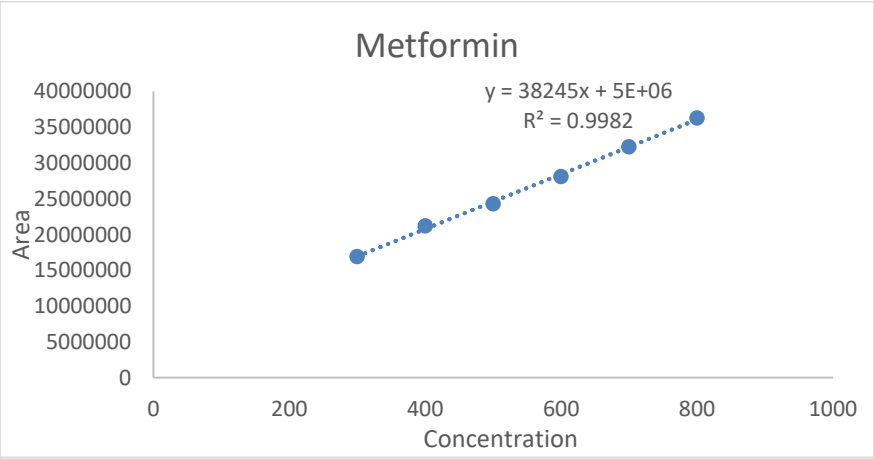
Linearity:

In the linearity studies, metformin hydrochloride, linagliptin and empagliflozin were dissolved in acetonitrile and added to the 55:45 acetonitrile to GAA solution at pH 3.2 to yield the desired concentrations ranging from 300 to 800 µg/mL for metformin hydrochloride and 1.5 to 4 µg/mL for linagliptin and empagliflozin. Six dilutions were made from each of the above concentrations and 20µl of each of the six dilutions was injected into the HPLC system and the results are shown in Table 2. A linear relationship between the concentrations and the peak areas were observed. Graphs 1, 2, and 3 are a linearity check plot of concentration versus peak areas used to help determine if the data is linear to perform linear regression.The correlation coefficient (R^2) for Metformin 0.9982 for Linagliptin 0.9977 and for Empagliflozin 0.9994 were found, which is in the acceptable ICH limits.

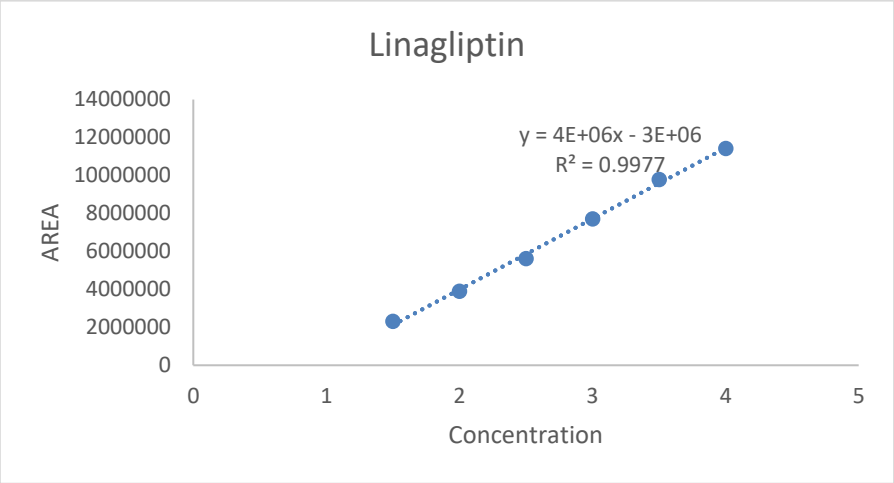
S.no	Metformin		Linagliptin		Empagliflozin	
	Conc(µg/ml)	Peak Area	Conc(µg/ml)	Peak Area	Conc(µg/ml)	Peak Area
1.	300	16909715	1.5	2312348	3	4949649
2.	400	21214903	2	3887064	4	9285665
3.	500	24288328	2.5	5622802	5	13334720
4.	600	28095544	3	7701041	6	17497390
5.	700	32250469	3.5	9781351	7	22329732
6.	800	36298492	4	11418426	8	26481928
R2	0.9982		0.9977		0.9994	

Table 2: linearity study results

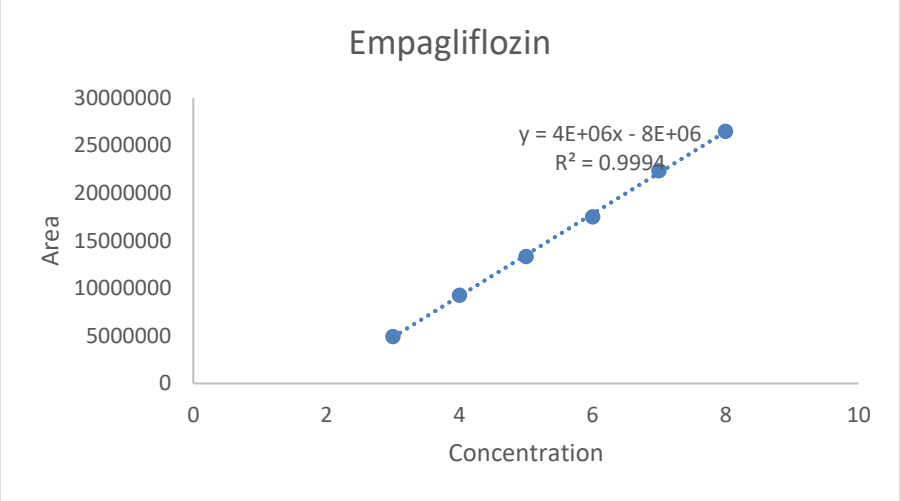
Graph 1: Linearity plot for Metformin



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Graph 2: Linearity plot for Linagliptin



Graph 3: Linearity plot for Empagliflozin

Precision:

Precision was checked by 6 replicates of drug solutions in a set, intra-day (Morning and Afternoon) and inter-day (Day-1, Day-2) and there was no much variation seen.

PrecisionMorning:

S.no	Metformin Area	LinagliptinArea	Empagliflozin Area
1	24289832	5622802	13331456
2	24282134	5621234	13337621
3	24283891	5621456	13339831
4	24280345	5629876	13338763
5	24287641	5622345	13337642
6	24287654	5626547	13330178
Average	24285250	5624043	13335915

Standard Deviation	3690.696	3443.842	4053.023
RSD(%)	0.01	0.06	0.03

Table 3: Precision Morning results

PrecisionAfternoon:

S.no	Metformin Area	Linagliptin Area	Empagliflozin Area
1	24289732	5628823	13334256
2	24282347	5626548	13332740
3	24289832	5627012	13335360
4	24281235	5629036	13336341
5	24287423	5625486	13338765
6	24287891	5621278	13330123
Average	24286410	5626364	13334598
Standard Deviation	3721.663	2838.25	2985.878
RSD(%)	0.015	0.05	0.02

Table 4: Precision Afternoon results

Precision Day 2:

S.no	Metformin Area	Linagliptin Area	Empagliflozin Area
1	24284562	5627643	13337329
2	24286751	5621234	13333456
3	24280965	5621456	13338752
4	24287643	5629876	13331873
5	24280972	5622345	13330832
6	24287659	5626547	13336537
Average	24284759	5624850	13334797
Standard Deviation	3145.477	3655.436	3198.435
RSD(%)	0.01	0.06	**0.02**

Table 5: Precision day 2 results

Precision Day 3:

S.no	Metformin Area	Linagliptin Area	Empagliflozin Area
1	24289832	5622802	13337642
2	24282134	5621234	13330934

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3	24283891	5621456	13337845
4	24280345	5629876	13336341
5	24287641	5622345	13338765
6	24287654	5626547	13330123
Average	**24285250**	**5624043**	**13335275**
Standard Deviation	**3690.696**	**3443.842**	**3765.938**
RSD(%)	**0.01**	**0.06**	**0.02**

Table 6: Precision day 3 results

LOD and LOQ:

Sample Name	LOD	LOQ
Metformin	3.75µg/ml	12.3 µg/ml
Linagliptin	0.75 µg/ml	2.475 µg/ml
Empagliflozin	0.375 µg/ml	1.23 µg/ml

Table 6: LOD and LOQ results

System suitability:

Parameter	Method			Acceptance Criteria
	Metformin Hydrochloride	Linagliptin	Empagliflizin	
Theoritical plates number per meter	3010	3446	3218	NLT 2000
Tailing Factor	1.286	1.102	1.348	NMT 2
Resolution	3.810		2.569	---

Table 7: System Suitability results

DISCUSSION

Concentrations of 300–800 µg/ml, 1.5–4 µg/ml and 3–8 µg/ml of metformin hydrochloride, linagliptin, and empagliflozin were used to assess linearity, respectively. A linearity check plot was made by plotting the concentration against the peak area which was to be used in a linear regression analysis. The values of the correlation coefficient (R2) were calculated as 0.9982, 0.9977 and 0.9994 for metformin, linagliptin and empagliflozin respectively following which the values are within acceptable limits set by ICH.

Validation of accuracy was carried out by the standard addition method at the levels of 80%, 100%, and 120%. The solutions were prepared in triplicates and tested using the developed method. The recoveries of the three drugs, metformin, linagliptin and empagliflozin, were between 99.98 and 100 percent, indicating that very little interference is caused by matrix components.

The strength of any procedure is the way it can withstand slight modifications of chromatographic conditions, as prescribed in the ICH guidelines. Robustness was assessed by changing the mobile phase flow rate by ±0.1 ml/min and the wavelength value of the lambda max of mobile phase by ±1 nm and system suitability was done in this study.

For the application of ICH guidelines, the limit of detection (LOD) and the limit of quantification (LOQ) were determined from the relationship between SNR. For calculating LOD and LOQ, signal-to-noise ratios of 3:1 and 10:1 were used,

respectively. The LOD and LOQ of the method were determined as 3.75 µg/mL and 12.3 µg/mL for metformin, 0.375 µg/mL and 1.23 µg/mL for empagliflozin and 0.75 µg/mL and 2.475 µg/mL for linagliptin.

CONCLUSION

In this study, new RP-HPLC method has been developed for the quantification of Metformin HCl, Linagliptin, Empagliflozin in bulk materials and pharmaceutical dosage forms which is highly sensitive, accurate, and precise. The limits for all the validation parameter are within ICH guidance. A very cost effective technique for identifying triple combination drugs from the formulations.

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