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Original Article

VALIDATED HPLC-UV METHOD FOR SIMULTANEOUS ESTIMATION OF LINAGLIPTIN AND EMPAGLIFLOZIN IN HUMAN PLASMA

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ABSTRACT

Objective: The proposed method aims to develop a simple, rapid, sensitive and validated isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of linagliptin and empagliflozin in human plasma.

Methods: Chromatography was performed on waters 2695 HPLC equipped with a quaternary pump. The separation was carried using discovery C18 (250×4.6×5) column, buffer: acetonitrile (68:32) as mobile phase with 1 ml/min flow rate. The analyte detection was monitored at 218 nm.

Results: Retention time of linagliptin, empagliflozin and internal standard was found at 6.421, 4.696, and 4.074 min respectively. The peaks were found to be free of interference. The method is validated over a dynamic linear range of 0.01-10.0 µg/ml for both drugs with a correlation coefficient of 0.998. The precision and accuracy of samples of six replicate measurements at lower limit of quantification (LLOQ) level were within the limit. The analytes were found to be stable in human plasma at-28 °C for 37 d.

Conclusion: The stability, sensitivity, specificity and reproducibility of this method make it suitable for the determination of linagliptin and empagliflozin in human plasma.

Keywords: Empagliflozin, Human plasma, Isocratic, Linagliptin, RP-HPLC

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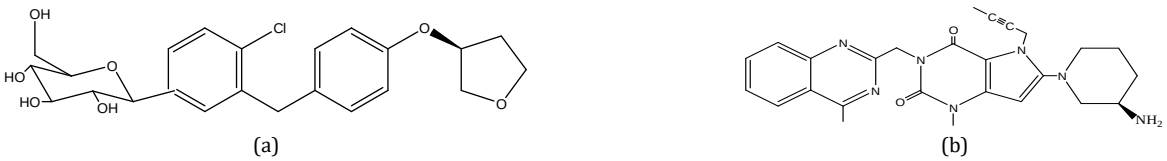
INTRODUCTION

Linagliptin (LIN) and empagliflozin (EMP) are used in the treatment of type-2 diabetes. The combination was marketed by under the trade name Glyxambi. The combination served as an adjuvant to diet and exercise to improve glycemia control in adults with type-2 diabetes who know to have the cardiovascular disease. General recommended dose is with 10 mg of empagliflozin and 5 mg of linagliptin once daily in the morning [1-3]. Linagliptin belongs to the class of drugs inhibiting the enzyme dipeptidyl-peptidase-4 (DPP-4). Chemically it is 8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methyl-quinazolin-2-yl) methyl] purine-2,6-dione. Linagliptin is soluble in methanol; sparingly soluble in ethanol; very slightly soluble in isopropanol and the solubility in water is<1 mg/ml [4, 5].

Empagliflozin, a sodium-glucose cotransporter 2, (SGLT2) inhibitor, is the newest class of oral hypoglycemic agent [6]. Chemically it is (2S,3R,4R,5S.6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxyphenyl] methyl] phenyl]-6-(hydroxymethyl) oxane-3,4,5-triol [7]. The structure of linagliptin and empagliflozin was given in fig. 1.



(a) (b)

Fig. 1: Chemical structure of empagliflozin (a), linagliptin (b)

The literature survey revealed that few methods like Ultra Performance Liquid Chromatography (UPLC) [8], High-Performance Liquid Chromatography (HPLC) [9, 10], Ultraviolet Spectroscopy (UV) [11, 12] were reported for estimation of the drugs individually orin combination other drugs.

A bioanalytical also reported for the estimation of empagliflozin and linagliptin by Liquid Chromatography-Mass Spectroscopy (LC-MS) [13] in dosage form etc. The review revealed the estimation of this combination was not performed in human plasma. Hence an attempt was made to develop a simple, rapid and reproducible RP-HPLC method for estimation in human plasma for this selected combination using telmisartan as an internal standard (IS). The developed method was validated as per USFDA [14] guidelines. The objective of present bioanalytical technique validation was to show that it is reasonable for the indented reason and will be useful for the pharmacokinetic studies.

Experiment

Reagents and chemicals

The pure drug samples of linagliptin and empagliflozin were purchased from Selleckchem LLC supplied by Pro lab marketing. HPLC grade Acetonitrile, HPLC grade Methanol and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study.

Instrumentation

Chromatography was performed with waters 2695 HPLC provided with a quaternary pump, high-speed autosampler, column oven, degasser and and 2996 PDA detector to provide a compact and with class Empower-2 software.

Chromatographic conditions

The separation was achieved by using discovery C18 (250×4.6µ×5 mm) column with a mobile phase consisting of 0.1% orthophosphoric acid and acetonitrile (68:32) with pH adjusted to 4.5. The separation was monitored for 10 min at 218 nm using 1 ml/min flow rate. The sample dilution was carried by using water: acetonitrile (50:50) ratio as diluent.

Preparation of internal standard

The working standard of internal standard was prepared by transferring 10 mg of telmisartan to the 10 ml volumetric flask and the volume was made by using diluent. From the resulting stock, 10µg/ml solution was prepared by further dilution.

Preparation of calibration and quality control solutions

The stock solutions of empagliflozin and linagliptin were prepared individual by dissolving 100 mg of the drug in 10 ml of diluent to obtain 10 mg/ml concentration each. The stock solutions were further diluted with diluent for spiking in plasma to obtain calibration curve standards. The spiking solutions for both analytes were prepared by transferring a varied amount to the 10 ml volumetric flasks and the volume was made by using diluent. The working concentration of both analytes was 0.01 to 10µg/ml. The calibration and quality control samples were obtained by spiking 10 µl of above-prepared solutions of each analyte to 250 µl plasma with 50 µl of the internal standard.

Sample preparation and extraction

The prepared spiking solution of analytes each 10 µl and 50 µl of internal standard was spiked into 250 µl of human plasma. To the spiked plasma, 2 ml of acetonitrile was added and vortexed for 2 min. The resulting solutions were centrifuged at 3200 rpm for 3 min. The resultant organic layer was used for analysis.

Methodology

A thorough and complete method of validation was performed following the USFDA guidelines. The method was validated for system suitability, autosampler carryover, specificity and screening of biological matrix, sensitivity, matrix effect, linearity, precision and accuracy, recovery of analyte and internal standard, ruggedness on precision accuracy and linearity, reinjection reproducibility and stability on day zero, freeze-thaw stability, LT at-28 °C and LT at-80 °C [15-17].

Specificity

Specificity and screening of biological matrix were assessed by using six blank standards and lower limit of quantification (LLOQ) level samples. All the samples were checked to determine the extent of interference contributed by plasma components with the analyte and internal standard.

Calibration curve

The Linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibration curve. The eight concentrations of the studied analytes range from 0.01 to 10µg/ml including LLOQ. The calibration curve is constructed by plotting the peak area ratio of the analytes to the internal standard against standard concentrations.

Accuracy and precision

Intra-day precision and accuracy were evaluated at lower, middle, high and lower limit of quantification quality control samples LQC, MQC, HQC and LLOQ in six replicates for both the analytes, while inter-day precision and accuracy were assessed for three consecutive days by using quality control samples. Mean values were obtained for calculated drug concentration over these batches. The accuracy and precision were calculated and expressed in terms of % mean accuracy and coefficient of variation (% CV), respectively.

Recovery

Recovery of the analytes from the extraction procedure was performed at LQC, MQC, and HQC levels. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

Sensitivity

Sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision (13). Sensitivity was done by LLOQ level sample in six replicates to know the lowest limit of detection, the % mean accuracy and % coefficient of variation was calculated.

Stability

Stability studies were performed as zero-hour, freeze-thaw, and long-term stability at-28 °C and at-80 °C. Day zero, Long-term stability at-28 °C and at-80 °C stability was carried out by using six replicates of HQC and LQC level of samples. The long-term stability of at-28 °C±5 °C was carried out by storing samples for 37d. The samples stored at-80 °C are thawed and analyzed immediately. The results obtained are compared with those obtained by freshly prepared samples. Whereas free-thaw stability was assessed by using LQC and HQC level of samples, the % mean accuracy and % coefficient of variation was calculated.

RESULTS AND DISCUSSION

Method optimization

To obtain best results for different mobile phase compositions containing buffer systems like acetate and phosphate with varied pH and organic solvents like methanol and acetonitrile were tried to provide adequate sensitivity and selectivity in short separation time. The best results were obtained with a mobile phase consisting 0.1% phosphoric acid (pH 4.5) and acetonitrile (68:32) with a flow rate of 1 ml/min. The detection was monitored at 218 nm. With these conditions the retention time of linagliptin, empagliflozin and telmisartan were obtained at 6.447, 4.716 and 4.079 respectively.

Method validation

System suitability and autosampler carry over

System suitability was performed by using the MQC level sample as six homogenous injections. The % coefficient of variation for retention time and response was calculated. The results were presented in table1. The obtained values are less than 1%, which shows the suitability of the system for the analysis of selected combination in human plasma.

Auto sample carryover was done by ULOQ (upper limit of quantification) and LLOQ level to ensure that it does not affect the accuracy and precision. There was no carryover observed.

Table 1: Statistical analysis of system suitability parameters

Parameter	IS	EMP	LIN	Acceptance
Retention time (t_R)	0.26	0.35	0.47	% RSD ≤ 2
Area under peak	0.40	0.30	0.51	% RSD ≤ 5
Resolution (R_s)	-	3.32	7.08	R_s>2
Number of theoretical plates (N)	10086.83	7073.67	10483.67	Increases with the efficiency of the separation.
Tailing Factor (T)	1.19	1.12	1.21	T ≤ 2
HETP (cm/plate)	0.0025	0.0035	0.0024	Smaller the value, higher the column efficacy

Specificity

Specificity demonstrates the ability of the method to unambiguously assess the analyte of interest in presence of other interfering peaks. No endogenous source of interference was observed at the retention time of analytes in all six lots of plasma when compared to blank and quality control samples. Typical chromatograms corresponding to blank and plasma sample spiked with the analyte and internal standard are given in Fig.2 and fig. 3 respectively. This shows specificity of method towards analytes.

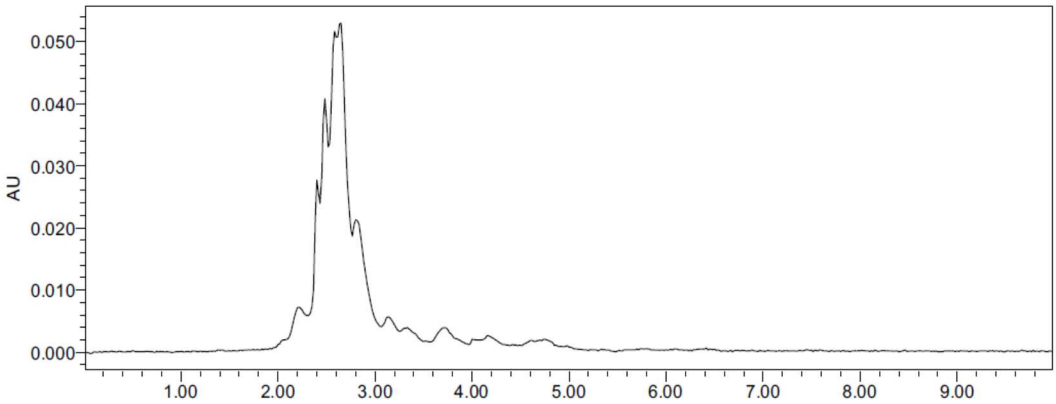


Fig. 2: Chromatogram of blank human plasma

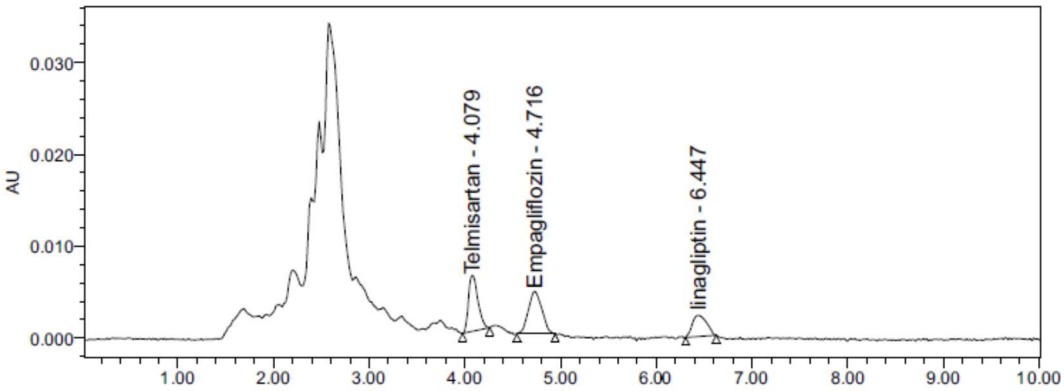


Fig. 3: Chromatogram of human plasma spiked with empagliflozin, linagliptin and telmisartan

Linearity

The ratio of peak area of the analyte to internal standard was used for the construction of calibration curve. The linearity of both analytes was established by eight-point calibration curve, concentration ranging from 0.01 to 10µg/ml. The most variable regression equation of the calibration curve for empagliflozin and linagliptin was y = 0.084x+0.01 and y = 0.0652x+0.0105 respectively. The linearity of the calibration graph was validated by the high value of the correlation coefficient with an average value of 0.999. The standard curve of empagliflozin and linagliptin are presented in fig. 4 and fig. 5.

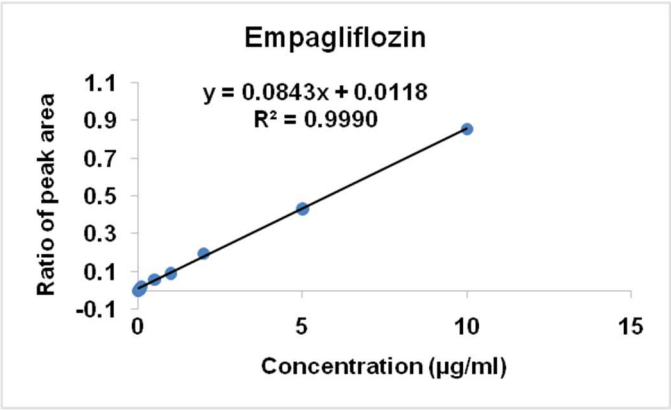


Fig. 4: Standard curve of empagliflozin

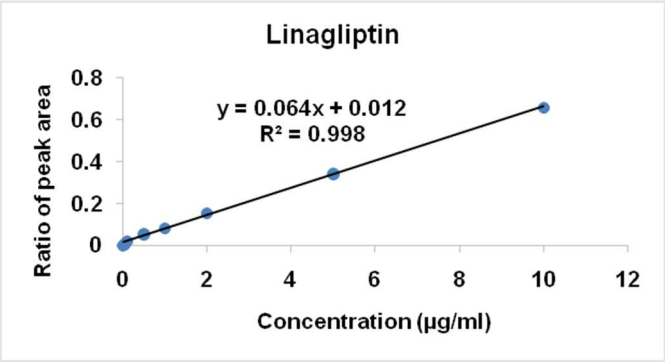


Fig. 5: Standard curve of linagliptin

Precision and accuracy

The precision and accuracy of the methods were assessed by analyzing six replicates of LLOQ, LQC, MQC and HQC levels. The accuracy of the method was determined by calculating % mean accuracy and the precision by calculating relative standard deviation (RSD). The data of precision and accuracy were summarized in table 2. The % mean accuracy of empagliflozin and linagliptin ranges from 98.00 to 101.82 and 98.00 to 101.23 respectively. The results obtained indicate an acceptable precision for all concentrations assayed for both intraday and interday samples.

Table 2: Intra and inter day precision and accuracy summary

Nominal conc.	Empagliflozin			Linagliptin		
(µg/ml)	mean±SD*	% CV	% mean recovery	mean±SD	% CV	% mean recovery
Between-batch						
5.00	5.016±0.2032	4.05	100.32	4.983±0.227	4.56	99.67
1.00	1.005±0.074	7.32	100.48	0.994±0.065	6.51	99.35
0.10	0.099±0.008	8.31	99.47	0.100±0.005	4.97	99.91
0.01	0.010±0.001	8.68	99.35	0.010±0.001	8.14	99.17
Day 1 (n=6)						
5.00	4.995±0.243	4.87	99.90	5.055±0.231	4.56	101.10
1.00	0.995±0.075	7.53	99.45	0.980±0.069	6.99	98.03
0.10	0.100±0.008	8.19	99.67	0.099±0.005	5.32	98.50
0.01	0.010±0.001	9.67	98.17	0.010±0.001	7.64	98.00
Day 2 (n=6)						
5.00	4.972±0.197	3.96	99.43	4.951±0.259	5.22	99.03
1.00	1.018±0.053	5.22	101.82	1.012±0.057	5.65	101.22
0.10	0.101±0.008	7.85	100.73	0.101±0.006	5.97	100.73
0.01	0.010±0.001	8.94	100.00	0.010±0.001	9.16	99.50
Day 3 (n=6)						
5.00	5.082±0.185	3.65	101.64	4.994±0.215	4.35	98.87
1.00	1.002±0.098	9.78	100.18	0.988±0.075	7.56	98.80
0.10	0.098±0.010	10.14	98.00	0.101±0.004	4.07	100.50
0.01	0.010±0.001	8.94	100.00	0.010±0.001	8.94	100.00

*Each value is represented as mean±SD of 6 observations (n=6), SD-Standard deviation, CV: Coefficient of variation.

Recovery

Recovery of empagliflozin and linagliptin was determined by comparing the mean peak areas of six replicates of three quality control samples (HQC, MQC and LQC) with the mean peak areas of unextracted quality control samples at the same level.

The result of recovery study is given in table 3. The results are within acceptance limit. The acceptable limit was % CV of recovery at each QC level and for IS should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %. The results obtained indicate the extraction efficiency of the optimized method.

Table 3: Extraction recovery data from human plasma

Analyte	Nominal concentration (µg/ml)	% Mean Recovery	% RSD[a]
Empagliflozin	0.1 (LQC)	65.03	0.34
	1.0 (MQC)	66.12	0.12
	5.0 (HQC)	55.16	0.66
	Across mean	62.102	9.72
Linagliptin	0.1 (LQC)	67.72	1.93
	1.0 (MQC)	62.04	0.37
	5.0 (HQC)	54.39	0.69
	Across mean	61.381	10.90
Internal standard	10	82.51	0.17

[a]n=6. % RSD-% Relative Standard Deviation

Ruggedness

The present method showed good ruggedness when the method was performed using different analyst and on the different instrument of same make. The results of ruggedness study were found to be within the acceptable limit, proving no significant analyst to analyst and instrument to instrument variation and hence ruggedness of the method.

Stability

The stability of the analytes in human plasma was evaluated by analysis six replicates of quality control samples at low and high concentration levels at room temperature over 24h(day zero). The measured concentrations were compared with that of freshly prepared and processed samples. The results obtained indicated that the two drugs empagliflozin and linagliptin were stable for at least 24hin human plasma when retained at room temperature. Freeze-thaw stability of the drugs in plasma samples were studied over three freeze-thaw cycles, by thawing at room temperature for 2–3h and refrozen for 12–24h. On the other hand, results obtained for quality control sample at-28 °C for 37 d and at-80 °C indicate the stability of analytes in human plasma. The results obtained are compiled in table 4.

Table 4: Stability data of empagliflozin and linagliptin in human plasma

Storage conditions	Empagliflozin		Lingaliptin	
	LQC	HQC	LQC	HQC
Day zero				
Mean calculated concentration (µg/ml)±SD*	0.10 1±0.008	4.951±0.221	0.098±0.007	5.021±0.111
%CV	7.80	4.51	7.05	2.02
% Mean accuracy	101.00	99.03	98.83	100.42
Freeze-thaw stability (3 cycles)				
Mean calculated concentration (µg/ml)±SD	0.097±0.007	4.975±0.238	0.096±0.005	4.961±0.170
%CV	7.12	4.78	5.73	3.44
% Mean accuracy	96.77	99.50	96.29	99.22
Stability at-28 °C				
Mean calculated concentration (µg/ml)±SD	0.099±0.003	5.012±0.104	0.101±0.008	5.088±0.095
%CV	2.75	2.09	7.95	1.88
% Mean accuracy	99.50	100.23	101.00	101.77
Stability at-80 °C				
Mean calculated concentration (µg/ml)±SD	0.009±0.001	0.097±0.007	0.957±0.072	4.974±0.237
%CV	7.08	7.12	7.52	4.78
% Mean accuracy	99.50	100.67	100.52	97.96

***** Each value is represented as mean±SD of 6 observations (n=6), SD-Standard deviation, CV: Coefficient of variation.

CONCLUSION

The proposed method for estimation of empagliflozin and linagliptin binary mixture in human plasma is a simple, accurate and reliable method. The single step protein precipitation, the short runtime of 10 min and isocratic elution makes the method economical and suitable for analysis of a large number of samples. The method is validated as per the requirement of US-FDA. It can be concluded that the method is suitable for routine quantification of empagliflozin and linagliptin in human plasma.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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