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Original research article

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Empagliflozin and Linagliptin in Solid Dosage Forms

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Abstract

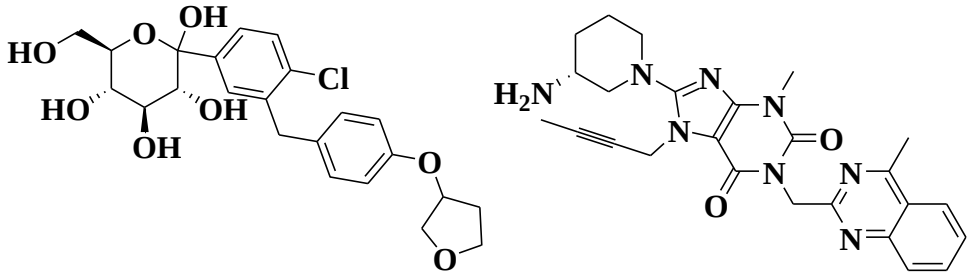
This study describes the development of an innovative, rapid, precise, selective and sensitive reverse phase high-performance liquid chromatography method for the quantitative determination of Empagliflozin (EMPA) and Linagliptin (LINA) in bulk and pharmaceutical dosage form as per International Conference on Harmonization (ICH) guidelines. In the present work, good chromatographic separation was achieved by isocratic method using a Thermo C_{18} column (250 mm ×4.6, 5µm) and a mobile phase consisting of acetonitrile: methanol in the ratio 50:50% v/v, at a flow rate of 1 ml/min. The effluents obtained were monitored at 280nm with the UV-visible detector. The calibration curves obtained were linear (r^2=0.999) over the concentration range of 5-25µg/ml and 1-5µg/ml for EMPA and LINA respectively. The retention time of EMPA and LINA was found to be 3.357± 0.3min and 4.085± 0.3min respectively. A run time of 7.0 minutes for each sample made it possible to analyze more than 200 samples per day. The limits of detection were 0.10 and 0.12µg/ml for LINA and EMPA respectively, and the limits of quantification were 0.25 and 0.40µg/ml for LINA and EMPA respectively. The high recovery values (99%-101%) indicate a satisfactory accuracy. The low percent relative standard deviation (% RSD) values in the precision study reveal that the method is precise therefore the method can be used for routine monitoring of EMPA and LINA in industry in the assay of bulk drug and dosage form.

Keywords: Empagliflozin, Linagliptin, RP-HPLC, Pharmaceutical dosage form, Method validation.

Introduction

Diabetes mellitus (DM) belongs to a category of metabolic disorder, characterized by chronic hyperglycaemia occurring due to deficiency in insulin secretion or action or both. People with type 2 DM are susceptible to various short term as well as long term complications including premature deaths and coma [1]. The combination of linagliptin and empagliflozin is on the market as tablets formulation for oral use for the management of type 2diabetes and cardiovascular risk. Empagliflozin (EMPA) is used as a sodium glucose cotransporter-2 (SGLT-2) inhibitor to improve glycemic control in adult patients with type 2 diabetes. SGLT-2 co-transporters reabsorb glucose from the glomerular filtrate in kidney and the glucuretic action resulting from inhibition of SGLT-2 which reduces renal absorption and lowers down the renal threshold for glucose, therefore increases glucose excretion which reduces hyperglycaemia and also helps in blood pressure reduction [2, 3]. Chemically EMPA is 1-chloro-4-(glucopyranos-1-yl)-2-(4-(tetrahydrofuran-3-yloxy)benzyl)benzene and having empirical formula is $C_{23}H_{27}ClO_7$ with molecular weight 450.91 g/mole (Fig. 1A). Linagliptin (LINA) is having competitive, reversible DPP-4 inhibitory action which is responsible for

DPP-4 breakdown reduction of GLP-1 and glucose-dependant insulinotropic polypeptide (GIP). From beta cells of the pancreas, GLP-1 and GIP stimulate the release of insulin during 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 [3-5]. Chemically LINA is (R)-8-(3-aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl- 1-(4-methylquinazolin-2-ylmethyl)-3,7-dihydro-purine- 2,6-dione and having empirical formula is $C_{25}H_{28}N_8O_2$ with molecular weight 472.5422 g/mole (Fig. 1B). Literature review revealed that few methods were described for the determination of EMPA and LINA alone or in combination with other drugs from pharmaceutical dosage forms and in human plasma including spectrophotometry [6-9], ultra-performance liquid chromatography (LC) [10], LC–mass spectroscopy [11], and high-performance LC (HPLC) [12-27] techniques. The aim of the present work is to develop and validate simple, fast and reliable reverse-phase HPLC method with ultraviolet (UV) detection for the simultaneous determination of EMPA and LINA in pure and pharmaceutical dosage forms. The proposed method can overcome the problems in all previously reported HPLC methods such as long time of analysis and expensive detectors.



(A) **(B)**

Figure 1 Chemical structure of (A) Empagliflozin and (B) Linagliptin

Materials and Methods

Instrumentation

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 binary pump for constant flow and constant pressure delivery and UV-Visible detector connected to software Data Ace for controlling the instrumentation as well as processing the generated data. Weighing was done on a Digital Micro Balance (CX-265) manufactured by Citizen Scale (I) Pvt. Ltd.

Reagents and chemicals

EMPA and LINA standard were obtained from Hetero drugs Ltd, Hyderabad, India. Methanol, acetonitrile were procured from Rankem, RFCL Limited, New Delhi, India. Ammonium acetate AR, sodium dihydrogen phosphate AR and ortho-phosphoric acid AR grade were procured from Central Drug House (P) Limited, New Delhi, India. The 0.45- mm pump nylon filter was obtained from Advanced Micro devices (Ambala Cantt, India). HPLC grade water was used throughout the study. Other chemicals used were of analytical or HPLC grade. Glyxambi Tab (10mg/5mg) was purchased from local market.

Chromatographic conditions

The isocratic mobile phase consisted of acetonitrile: methanol in the ratio 50:50% v/v, flowing through the column at a constant flow rate of 1.0 ml/ min. The mobile phase was filtered through nylon 0.22 µm membrane filters and was degassed before use (30 min). A Thermo (C-18) column (5 µm, 250mm x 4.60mm) was used as the stationary phase. By considering the

chromatographic parameter, sensitivity and selectivity of method for drugs, 280nm was selected as the detection wavelength for UV-Visible detector.

Selection of mobile phase

Initially to estimate EMPA and LINA simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was acetonitrile: methanol in the ratio 50:50% v/v, run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by Sonication. Flow rate employed for analysis was 1 ml/min.

Selection of diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials methanol was used as diluents.

Preparation of stock solution

Accurately weighed 10 mg API of EMPA and LINA was transferred into 10 ml volumetric flask separately and added 5ml of methanol as diluents, sonicated for 20 minutes and volume was made up to 10ml with methanol to get concentration of solution 1000µg/ml (Stock-A)

Preparation of sub stock solution

5 ml of solution was taken from stock-A of both the drug and transferred into 50ml volumetric flask separately and diluted up to 50 ml with diluent (methanol) to give concentration of 100µg/ml of EMPA and LINA respectively (Stock-B).

Preparation of different solution

0.5ml, 1.0ml, 1.5ml, 2.0ml and 2.5ml of stock-B were taken separately in 10 ml volumetric flask and volume was made up to 10ml with (methanol). This gives the solutions of 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml, for EMPA. In same manner 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml and 5µg/ml of LINA also prepared.

System suitability parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min. After complete saturation of column, six replicates of working standard of EMPA 10µg/ml for EMPA and 5µg/ml LINA was injected separately. Peak report and column performance report were recorded for all chromatogram.

Linearity and calibration graph

To establish the linearity of analytical method, a series of dilution ranging from 5-25 µg/ml for EMPA and 1-5µg/ml for LINA were prepared. All the solution were filtered through 0.45µm membrane filter and injected, chromatograms were recorded at 280.0 nm and it was repeat for five times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

Stability study

Samples prepared for repeatability study were preserved for 24 h at room temperature and analyzed on the following day to test for short-term stability.

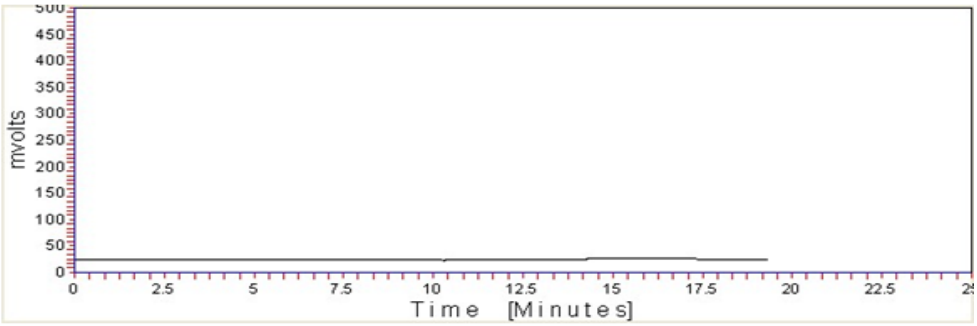
Analysis of both the drug in tablet Sample

Twenty tablets were accurately weighed and their mean weight was determined. The tablets were grinded to fine powder, an accurately weighed quantity of powder equivalent to 10 mg of EMPA and 5mg of LINA was transferred to 10 ml volumetric flask containing methanol. The solution was sonicated for 25 min and the final volume was made with mobile phase. The mixture was then filtered through a 0.45 µm filter. The stock solution was further diluted sufficiently with methanol to get sample solution of drug concentration of 10µg/ml EMPA and 5µg/ml LINA respectively. The amounts of EMPA and LINA in tablets formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with formulation.

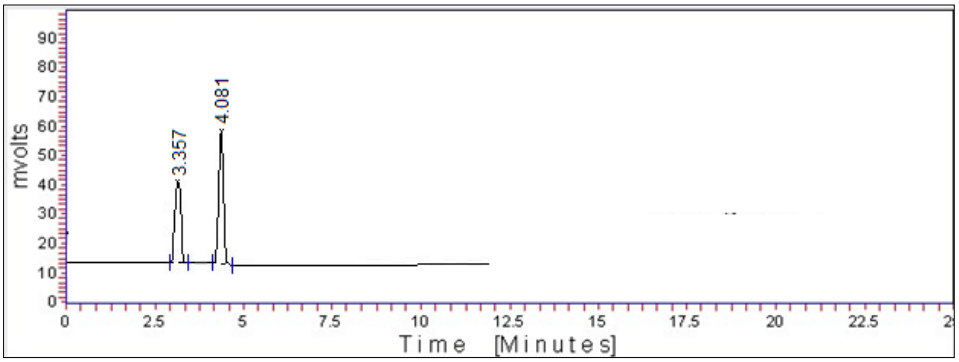
Results and discussion

Chromatography

The mobile phase was chosen after several trials with methanol, isopropyl alcohol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of methanol: acetonitrile (50:50v/v) was selected to achieve maximum separation and sensitivity. Flow rates between 0.5 and 1.5 min were studied. A flow rate of 1 ml/min gave an optimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase C_{18} column, the retention time of EMPA and LINA was found to be 3.357± 0.3min and 4.085± 0.3min respectively. Total time of analysis was less than 7min. The maximum absorption of EMPA and LINA was detected at 280 nm and this wavelength was chosen for the analysis Fig. 2. The developed method was validated for system suitability, linearity, sensitivity, precision, accuracy, robustness selectivity, and specificity and is applied for forced degradation studies as per the ICH guidelines [28].



(A)



(B)

Figure 2 Chromatograms of (A) Blank mobile phase (B) EMPA and LINA (15,3µg/ml) as reference substances

System suitability

System suitability parameters such as number of theoretical plates, HETP and peak tailing are determined. The results obtained are shown in Table 1. The number of theoretical plates for EMPA and LINA was 2639.833 and3169.833.

Table 1 Results of system suitability parameters

Parameters	**% MEAN±SD***	
	EMPA	**LINA**
No. of Theoretical Plates	2639.833±38.53	3169.833±30.7208
Tailing Factor	1.047±0.024	1.147±0.0216
Retention time	3.350±0.0053	4.0855±0.0031

Linearity

The calibration curve was linear over the concentration range of 5-25 and 1-5µg/ml for EMPA and LINA. The linearity was represented by a linear regression equation as follows:

Y (EMPA) = 43.57conc+6.888 (r^2 = 0.999)

Y (LINA) = 252.8conc+5.372 (r^2 = 0.999)

Accuracy

Method accuracy was performed by adding known amounts of EMPA and LINA to the preanalysed tablet solution and then comparing the added concentration with the found concentration. Three levels of solutions were made which correspond to 80%, 100% and 120% of the nominal analytical concentration (15, 3µg/ml for EMPA and LINA). Each level was made in triplicate Table 2. The mean percentage recoveries obtained for EMPA and LINA was 98.77-99.12% and 98.48-99.03% respectively and RSD was less than 2.

Table 2 Results of recovery study

% LEVEL	**% MEAN±SD***	
	EMPA	**LINA**
80%	98.77±0.294	99.03±0.241
100%	99.12±0.601	98.48±0.421
120%	99.04±0.452	98.98±0.962

***** Value of three replicate and three concentrations.

Precision

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 5, 10, 15, 20 and 25µg/ml for EMPA and 1, 2, 3, 4 and 5µg/ml for LINA indicates the precision under the same operating condition over short interval time and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Intermediate precision

Five dilutions in three replicates were analyzed on two different days and by two analysts for day-to-day and analyst-to-analyst variations and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Robustness

As per ICH norms, small but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, acetonitrile: methanol (50:50 % v/v) to (45:55 % v/v). Results of robustness are reported in table 3.

Table 3 Results of precision

PARAMETER	% MEAN±SD*	
	EMPA	LINA
Repeatability	99.361±0.054	98.245±0.044
Day To Day	99.055±0.065	97.347±0.044
Analyst to Analyst	99.527±0.045	98.165±0.030
Robustness	99.232±0.063	97.542±0.056

***** Value of five replicate and five concentrations

Detection limit and quantitation limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve table 4.

Table 4 Results of LOD and LOQ

Name	LOD (μg/ml)	LOQ (μg/ml)
EMPA	0.12	0.40
LINA	0.10	0.25

Analysis of tablets

The concentration of EMPA and LINA in the tablets formulation was found to be 99.7 and 98.40%. The low values of % RSD indicate that the method is precise and accurate in Table 5.

Table 5Analysis of tablet formulation

	EMPA*	LINA*
Label Claim (mg)	**10mg**	**5mg**
% Found (mg)	9.97	4.92
% Assay	99.7	98.40
% RSD	0.045	0.063

*Average of three determination

Conclusion

The proposed HPLC method was validated as per the International Conference on Harmonization (ICH) Q2B Guidelines and was found to be applicable for routine quantitative analysis of EMPA and LINA by HPLC in pharmaceutical dosage form. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of azilsartan with no interference from other formulation excipients. The proposed method was highly reproducible, reliable, rapid, robust and specific. Therefore, a high percentage of recovery and the run time of less than ten minutes allow its application for the routine determination of EMPA and LINA in the pharmaceutical dosage form.

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