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NEW VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF EMPAGLIFLOZIN IN HUMAN PLASMA

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Keywords:

Empagliflozin, Human plasma, method development, Validation

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ABSTRACT: A new reversed phase simple, economic and specific validated high performance liquid chromatography method for the estimation of empagliflozin in human plasma is presented. A chromatographic separation was carried out isocratically by mobile phase comprising of methanol and acetonitrile, 50:50% v/v at a flow rate of 1.0 ml/min on agilent long C18 column (250 mm × 4.6 mm, 5 µm) with photodiode arrays (PDA) detection at 270 nm. The bioanalytical procedure involves deproteination of plasma with 30% ethyl acetate and liquid-liquid extraction process. Chromatogram showed a peak of empagliflozin at retention time of 8.898 min. The correlation coefficient (r^2) was found to be 0.999 in the concentration range of 50-150 µg/ml. No interference peak was observed in blank plasma samples at the retention time of empagliflozin. The percentage of relative recovery and coefficient of variation (CV %) values of precision and accuracy were within the acceptable limits. The method proved simple, cost effective and sensitive for estimation of empagliflozin in human plasma.

INTRODUCTION: Diabetes is one of the largest world health problems of the 21st century. There is a need for drugs with novel mechanisms of action that can be used alone or in combination with other anti-diabetic agents to improve glycemic control and hyperglycemia is often poorly controlled despite a number of therapeutic options [1 - 4]. Unlike previously available agents, sodium-glucose co-transporter 2 (SGLT2) inhibitors offer an insulin-independent mechanism for maintaining blood glucose levels, since they promote urinary glucose excretion (UGE) by inhibiting glucose reabsorption in the kidney.

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Empagliflozin is a member of the SGLT2 inhibitor class of drugs being investigated for the reduction of blood glucose levels in adults with T2D [5 - 9].

The IUPAC name of empagliflozin is (1-chloro-4-[b-d-glucopyranos-1-yl] - [4-([s] -tetrahydrofuran-3-yl-Oxy) benzyl] -benzene **Fig. 1**. The empirical formula is $C_{17}H_{19}ClO_2Si$ and the molecular weight is 455.91. Empagliflozin is a white to yellowish, non-hygroscopic crystalline solid. It is commercially marketed under the name Jardiance and marketed by Boehringer Ingelheim International GmbH, Ingelheim, Germany [9 - 12]. The drug was approved by USFDA on August 1st 2014 and in Europe in May 2014 [10 - 12].

Empagliflozin is not official in any pharmacopoeia. A literature survey on empagliflozin revealed that, until now only few analytical methods were reported for its estimation of empagliflozin such as UV-visible spectroscopy [13], HPLC method [14 - 17] in

bulk and API form. To date, there is no RP-HPLC method reported for determination of empagliflozin in any preclinical species and human plasma. Hence, we felt that there is a great need to develop and validate a simple, specific and reproducible, economical LLE-RP-HPLC method for estimation of empagliflozin in human plasma.

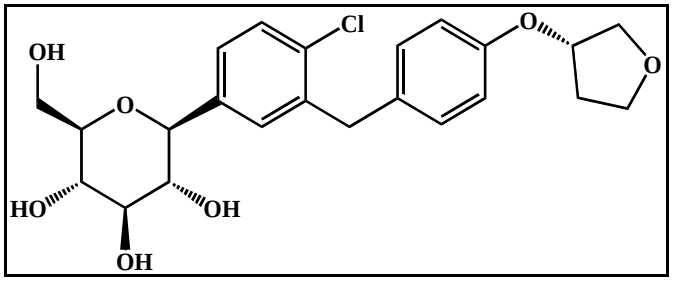

FIG. 1: CHEMICAL STRUCTURE OF EMPAGLIFLOZIN

MATERIALS AND METHODS:

Chemicals and Reagents: The empagliflozin authentic sample was kindly provided by Spectrum laboratories, Hyderabad, Telangana, India. HPLC grade acetonitrile and methanol were procured from Rankem, Ranbaxy fine chemicals limited, New Delhi, India. Blank human plasma was procured as a gift sample from the nilourfer hospital, Hyderabad, India. The 0.45 µm nylon membrane filters were purchased from Pall India Pvt. Ltd. Mumbai, India.

Instrumentation and Chromatographic Conditions: The analysis was performed using waters 2695 binary pump with 2996 photo diode array detector used for method development, plasma studies and method validation. The output signal was checked and the acquisition and integration of data was performed using Empower software on a Pentium (Digital equipment Co) computer. A chromato-graphic separation was carried out isocratically by mobile phase comprising of methanol and acetonitrile, 50:50% v/v at a flow rate of 1.0 ml/min on agilent long C18 column (250 mm × 4.6 mm, 5 µm). The mobile phase was pre-mixed, filtered through a 0.4 µm nylon filter and degassed. The flow rate was kept at 1.2 mL. Throughout the LC column oven was maintained at 35 ºC and detection was monitored at 270 nm. The injection volume was 20 µL.

Preparation of Solutions:

Preparation of Standard Solution: Accurately weighed 10 mg of empagliflozin was transferred into 10 ml volumetric flask, dissolved and made up to the mark with diluent. This was a solution having strength of 1000 µg/ml of empagliflozin. From this solution, 0.2 ml of the solution was pipetted out and diluted up to 10 ml to get 20 µg/ml of empagliflozin.

Extraction of Plasma from Blood: Blood was collected into an EDTA containing tube and then it was centrifuged for 10 min at 3000 rpm. Blood was separated into layers after centrifugation. The supernatant which contains stray yellow colour (plasma) was collected and used for sample preparation.

Preparation of Plasma Solution: 0.5 ml plasma sample was deproteinated with 20 µL of 30% ethyl acetate and to this 400 µL of empagliflozin was added and the contents of the tube were mixed on a vortex mixture for 3 min. The tubes were kept in an inclined position on a reciprocating shaker at 100 strokes per min for 30 min. The mixture was vortex-mixed for one min and centrifuged at 4000 rpm for 20 min.

The supernatant was transferred to polypropylene tube and washed with one ml of ethyl acetate by mixing for one min and centrifuging at 1000 rpm for 2 min at 5 °C. The supernatant layer of ethyl acetate was discarded and an aliquot of 20 µL was injected to HPLC for analysis using the optimized chromatographic conditions.

Method Validation: ICH Guidance for industry was followed for validation of the method. Selectivity, linearity, accuracy, precision (within a run and between run), LLOQ and stability were assessed during method validation [18].

System Suitability: System suitability is used to confirm the suitability for the theoretical plates, tailing factor and reproducibility of the RP-HPLC system. Freshly prepared solutions of empagliflozin 20 µg/mL were injected into RP-HPLC system.

Selectivity: Selectivity was observed by comparing the chromatograms of spiked sample and drug free plasma samples. For this purpose spiked sample of empagliflozin (20 µg/mL) and blank plasma samples from 6 different sources were prepared and injected.

Linearity: Calibration standard solutions were prepared in plasma from the working solution. Five calibration curves ranging from 50 to 150 µg/mL were run to establish the linearity by using linear regression analysis. Linearity of empagliflozin was observed in term of coefficient of determination (r^2). The concentration of empagliflozin in each calibration curve, the percentage relative recovery and CV (% RSD) were determined.

Accuracy and Precision: Quality control samples (n=5) were prepared at four different levels HQC, MQC, LQC and LLOQ analyzed subsequently to evaluate with-in run accuracy and precision. The concentration of empagliflozin was calculated from a standard calibration curve that was concurrently obtained. Accuracy was analyzed at each level by comparing the observed concentration with the nominal concentration as a mean relative percentage recovery, while precision was observed in terms of % CV.

Stability: Stability of empagliflozin in plasma was observed at HQC and LQC levels. To evaluate stability at room temperature, 5 replicates of both levels were prepared in plasma and stored at room temperature for 24 h freeze and thaw stability was observed after four and thaw cycles. The sample was frozen after each cycle for at least 24 h before thawing. For long period stability HQC and LQC were prepared and stored at -20 ºC for two months.

RESULTS AND DISCUSSION:

Method Development and Optimization of Chromatographic Conditions: Empagliflozin was scanned between the wavelength of 200-400 nm and showed maximum absorbance at 270 nm so it was selected as detection wavelength shown in **Fig. 2.**

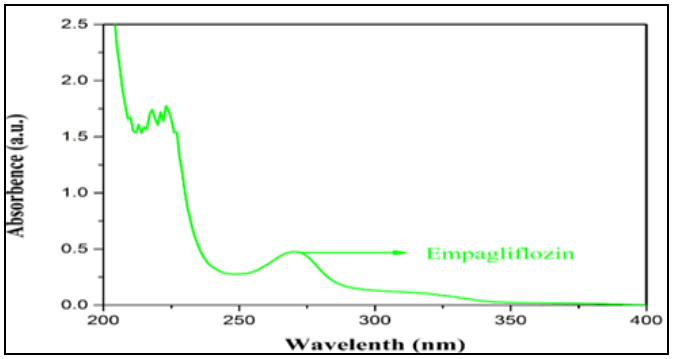

FIG. 2: UV SPECTRA OF EMPAGLIFLOZIN

Optimized Method: In this work to achieve good separation and peak shapes, column like Agilent long C18 column (250 mm × 4.6 mm, 5 µm) having more surface area was tried and there is an improvement in resolution and peak shapes was observed. As the analysis of compounds was needed different mobile phases like phosphate buffer, formic acid and orthophosphoric acid were tried with different pH conditions from 2.0 to 7.0. But, organic solvents like methanol and acetonitrile were showing good separations and less column back pressure. Finally, the best results were obtained by mobile phase composition of methanol and acetonitrile in the ratio of (50: 50 V/V) with flow rate of 1.2 mL/min was selected at the oven temperature of 35 °C methanol and acetonitrile were used as diluents.

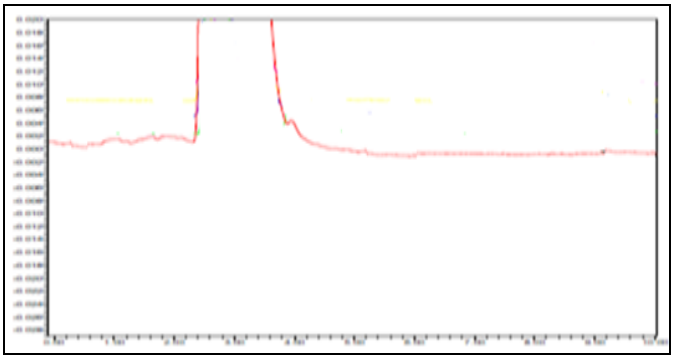

FIG. 3: TYPICAL HPLC CHROMATOGRAM OF BLANK PLASMA

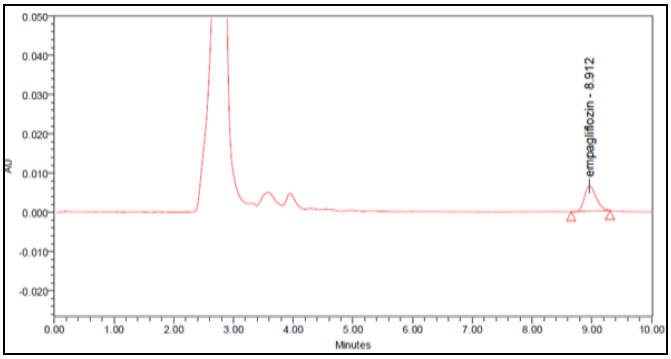

FIG. 4: TYPICAL OPTIMIZED HPLC CHROMATO-GRAM OF EMPAGLIFLOZIN

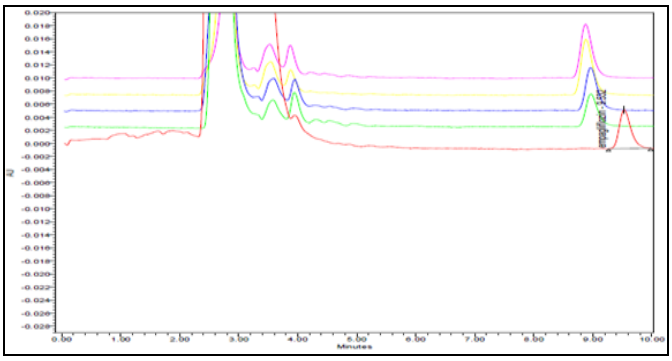

FIG. 5: OVERLAY CHROMATOGRAM OF PLASMA SPIKED WITH EMPAGLIFLOZIN 50-150 µg/mL

The developed RP-HPLC method was found to be highly specific. The representative chromatograms of blank plasma, optimized standard chromatogram and overlay standard chromatogram were summarized in **Fig. 3, 4** and **5**.

Method Validation:

System Suitability Parameters: System suitability method acceptance criteria set in each validation run were: tailing factor ≤ 2.0 and theoretical plates > 2000. In all cases, the relative standard deviation (RSD) for the analytic peak area for two consecutive injections was < 2.0%. Results are tabulated in **Table 1**.

TABLE 1: SYSTEM SUITABILITY PARAMETERS

Name	Retention time (min)	Tailing factor (T_f)	Theoretical plate (N)
Empagliflozin	2.172	1.48	5342

Selectivity: All the injections were processed at the wavelength provided in the method. There was no interference observed from diluents blank solutions, excipients blend solutions with empagliflozin peak. Empagliflozin was eluted at 8.898 min. Hence this method is selective.

Linearity: Linearity graph of average area at each level against the concentration in ppm is plotted and is found to be a straight line graph. The calibration curves (n=5) were linear with $r^2 \geq 0.999$ over the range of 50-150 µg/ml.

TABLE 2: LINEARITY DATA OF EMPAGLIFLOZIN

Concentration (µg/mL)	Peak area (µV* sec)
0	0
50	41915
75	63467
100	85134
125	109926
150	132956
Correlation coefficient	0.999
Slope	1691.7
Y-Intercept	887.1

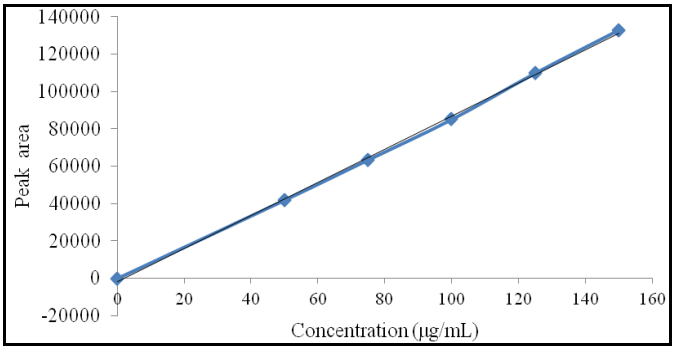

FIG. 6: CALIBRATION CURVE OF EMPAGLIFLOZIN

The value of the slope was 1691.7 and the intercept was 887.1. The correlation coefficient is found to be more than 0.999. The linearity results were expressed in **Table 2** and the linearity graph was shown in **Fig. 6**.

Accuracy and Precision: The mean percentage of relative recovery for within run precision and accuracy were 99.3 % and 103.1% while % RSD ≤ 4.00%. The mean percentage of relative recovery for between run precision and accuracy were 97.6% and 99.5% whereas % RSD ≤ 3.47%. The method has shown consistent and high mean recovery ranging from 98.90 - 99.22%. The precision and recovery results were expressed in **Table 3**.

TABLE 3: ACCURACY, PRECISION DATA OF EMPAGLIFLOZIN

S. no.	Concentration (ng/mL)	Concentration measured (Mean ± S.D)	CV %	% Accuracy
Within run[a] (n=5)	0.25 (LLOQ)	0.26 ± 0.001	0.38	101.2
	0.5 (LQC)	0.575 ± 0.02	4.00	99.3
	25 (MQC)	25.95 ± 0.212	0.81	103.1
	50 (HQC)	50.65 ± 0.77	1.52	100.2
Between run[b] (n=5)	0.25 (LLOQ)	0.001 ± 0.26	0.38	99.5
	0.5 (LQC)	0.56 ± 0.07	3.47	98.6
	25 (MQC)	25.45 ± 0.494	1.96	97.6
	50 (HQC)	53.2 ± 0.7	1.31	98.6

[a]Analysed on same day, [b]Analysed on five different days

Stability: Empagliflozin proved stable at room temperature for 24 h with a mean percentage recoveries for HQC and LLOQ at 101.5% and 103.5 %, respectively, and a CV≤ 4.08 %. After 4 freeze and thaw cycles, the mean percentage recoveries were 99.6% and 100.0%, respectively, and a CV≤ 2.0%.

TABLE 4: STABILITY STUDIES OF EMPAGLIFLOZIN

S. no.	Concentration (ng/mL)	Concentration measured (Mean ± S.D)	CV %	% Accuracy
Short term stability (n=5)	50 µg/mL (HQC)	49.35 ± 1.34	2.71	101.5
	0.5 µg/mL (LLOQ)	0.49 ± 0.02	4.08	103.5
Freeze –thaw stability (n=5)	50 µg/mL (HQC)	50.8 ± 0.7	1.37	99.6
	0.5 µg/mL (LLOQ)	0.51 ± 0.01	2.00	100.1
Long term stability (n=5)	50 µg/mL (HQC)	50.8 ± 0.70	1.37	98.6
	0.5 µg/mL (LLOQ)	0.5 ± 0.01	2.00	98.2

For long-term stability empagliflozin also proved stable with mean percentage relative recovery of 98.2% and 98.6% respectively for HQC and LLOQ

while CV≤ 2.0%. Stability data were tabulated in **Table 4**.

CONCLUSION: This new validated RP-HPLC method for the estimation of empagliflozin in human plasma is accurate, precise, less cost and reproducible. The method described, does not require expensive solvents, chemicals and reagents. It does not involve complex sample preparation or complicated instrumentation. All the validation parameters were within the acceptable limits for estimation of empagliflozin in human plasma. The proposed method can be used for predictable bioanalysis of empagliflozin in various biological samples to support bioequivalence and pharmacokinetic studies.

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CONFLICT OF INTEREST: The authors did not report any conflict of interest.

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