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Development and validation of empagliflozin in human plasma using nevirapine as internal standard by liquid chromatography-tandem mass spectrometry

Mogalipuvvu Jagadeesh
Department of Chemistry, Bhagwant University, Ajmer, Rajasthan, India-305004

Gajendra Kumar
Department of Chemistry, Bhagwant University, Ajmer, Rajasthan, India-305004 | Department of Chemistry, FoE, Teerthanker Mahaveer University, Moradabad, (UP), India-244001
*Corresponding author email: gaj25chem@gmail.com

***Abstract*---**The present LC-MS/MS method for the estimation of Empagliflozin in human plasma by using Nevirapine as an internal standard was established and validated as per ICH guidelines. The best response was obtained with Phenomenex Synergi,4µm,4.6×75mm, and a mobile phase containing a mixture of 5mM Ammonium Acetate buffer with 0.1% Formic Acid: Acetonitrile (50:50 v/v) was delivered at a flow rate of 0.900 mL/min by positive ion mode (API 4000Q Trap) with an injection volume of 20 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is m/z451.52>71.32 for Empagliflozin and m/z 267.3>226.8 for Nevirapine (Internal standard) were used for quantization. The retention time of Empagliflozin and Nevirapine (Internal standard) was found to be 1.50 min and 2.40 min respectively. Linearity was established for Empagliflozin in the range of 2.0pg/mL to 979.9pg/mL with a correlation coefficient (r=0.999) and the overall percentage recovery was 94.63 % for Empagliflozin and 91.94 % for Nevirapine (Internal standard) respectively. The CV % values of accuracy and precision for Empagliflozin were found to be ≤ 15 %, which indicates the accuracy and precision of the proposed method. The CV % values of accuracy and precision of Empagliflozin for stability studies were found to be ≤ 15 %, which indicates the stability of the proposed method.

***Keywords*---**empagliflozin, nevirapine, ammonium acetate buffer, acetonitrile.

Introduction

Empagliflozin is a sodium-glucose co-transporter-2 (SGLT-2) inhibitor to improve glycemic control in adult patients with type 2 diabetes. (N. Padmaja et.al 2006) Chemically known as (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3-yloxy] phenyl} methyl) phenyl]-6-(hydroxyethyl) oxane-3,4,5-triol. (Potdr Ashwini et.al 2019) The Molecular Formula of Empagliflozin C23H27ClO7 and its molecular weight is 450.91g/mol .(Manoj ,JyothiVikhe et.al 2018) The chemical structure of Empagliflozin is shown in Fig.1.

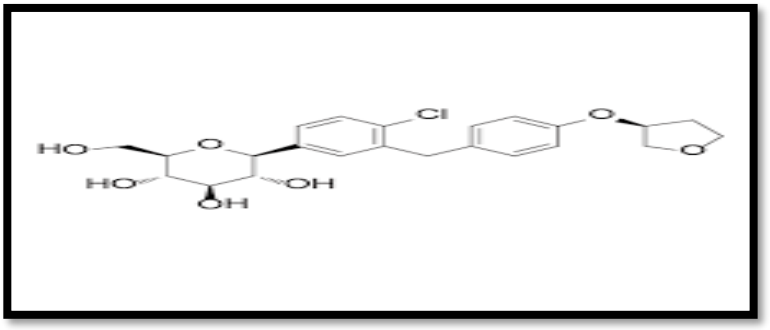

Fig 1. Structure of Empagliflozin

A literature review reveals that very few methods were reported for the estimation of Empagliflozin individually and combined with other drugs at the time of commencement of work. However, there is no work was reported for the estimation of Empagliflozin in human plasma by LC-MS/MS method at the time of commencement of work. Hence, in the present study, an attempt has been made by the author to develop a rapid and reliable analysis in bio-analytical laboratories; a new method for the determination of Empagliflozin in human plasma within a short time of analysis is described in this work**. (**Patel et.al to phanindra et.al)

Materials and Methods

Apparatus & Equipment

An LC-MS/MS method was performed on a liquid chromatographic system consisting of Mass Lynx 4.1 SCN805, an auto sampler of Shimadzu (SIL-HTC) coupled with an API 4000 Trap triple quadrupole mass spectrometer with electrospray ionization (ESI) used for analysis and Mass Lynx 4.1 SCN805Analyst software (version 1.4.2) for processing and data collecting. Phenomenex Synergi,4µm(4.6×75mm)Column is used as a stationary phase. An ultrasonic bath sonicator(Frontline FS 4, Mumbai, India), semi-micro analytical balance (India) and Whatman filter paper No.1 is used in the study.

Reagents & chemicals

Empagliflozin were procured from Aurobindo Laboratories, Hyderabad. Nevirapine (Internal Standard) was procured from Aurobindo Laboratories Hyderabad. Acetonitrile of HPLC grade was procured from Chemical Laboratory Hyderabad. The water of HPLC grade was obtained from Ammonium phosphate and orthophosphoric acid of HPLC grade was procured from

Preparation of mobile phase
Preparation of 5mM Ammonium acetate with 0.1% orthophosphoric acid in water

To approximately 500ml of water added 0.7708g of ammonium acetate, made up the volume to 2000ml with water, mixed well, and sonicated. To the above buffer added 2.000ml of Formic Acid. Labeled and stored the solution at ambient temperature. Recorded the details in Buffer preparation form.

Preparation of Mobile Phase

To 500ml of above buffer added 500ml of Acetonitrile, mixed well, and sonicated. Labeled and stored the solution at ambient temperature. Then recorded the details in the Mobile phase preparation form.

Preparation of standard and working solutions for Empagliflozin

The Empagliflozin stock solution was prepared by dissolving 10 mg of Empagliflozin in 1% ammonia solution in acetonitrile and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/mL. This solution was kept in a refrigerator at 2-8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into a plasma to obtain calibration curve standards and quality control samples for further use. All other dilutions were made in the mobile phase.

Preparation of stock solution for Nevirapine (Internal standard)

A stock solution of Nevirapine (Internal standard) was prepared by dissolving 10 mg of Nevirapine in diluents (mixture of HPLC grade acetonitrile and water in a ratio (60:40, v/v) and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/mL. This solution was kept in the refrigerator at 2-8 °C. Working IS solutions were prepared by suitably diluting the above-mentioned stock solution fresh before use.

Preparation of calibration curve standards and quality control (QC) samples

A calibration curve standard consisting of a set of eight non-zero concentrations ranging from 2.0pg/mL to 979.9 pg/mL of Empagliflozin was prepared. Prepared quality control samples consisted of concentrations of 5.5pg/mL (lower quality control sample), 480.0pg/mL (middle quality control sample) and 780.0 pg/mL (higher quality control sample) for Empagliflozin. These samples were stored at -

70 °C ± 10 °C until use. Twelve sets of LQC and HQC samples were stored at -20 °C ± 5 °C to check stability.

Preparation of plasma samples

For the preparation of plasma samples, human blood samples were collected into polypropylene tubes containing K_2-EDTA. Each tube was centrifuged for 15 min at 8500 rpm and the supernatant was collected in another tube. To the supernatant 1 mL of acetonitrile was added and kept for 10 min for the plasma proteins to precipitate and then the supernatant was collected for further use

Preparation of sample solution

After bulk spiking, aliquots of 100µL for calibration curves and 100 µL for quality controls of spiked plasma samples were pipette out into a pre-labeled polypropylene micro-centrifuge tubes and then all the bulk spiked samples were stored in the deep freezer at -70 °C ± 10 °C, except twelve replicates each of LQC and HQC, which were stored in -20 °C ± 5 °C for generation of stability data. The thawed samples were vortexes to ensure complete mixing of the contents.

Selectivity and Sensitivity

Selectivity was performed by analyzing the human blank plasma samples from six different sources (donors) A sensitive bioanalytical method development and validation of Empagliflozin in human plasma by LC-ESI-MS/MS with an additional hemolysed group and lipedimic group to test for interference at the retention times of analytes. The sensitivity was compared with the LLOQ of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of LOQ of Empagliflozin and 5% of the mean peak area of Nevirapine.

Precision and accuracy

It was determined by replicate analysis of quality control samples (n = 6) at LLOQ (lower limit of quantification), LQC (low quality control), MQC (Medium quality control), HQC (high quality control) and ULOQ (upper limit of quantification) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect

The matrix effect due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (LLE) with the reconstitution samples extracted blank plasma sample spiking with the analyte. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (%CV) of ≤ 15%.

Recovery

The extraction recovery of Analyte and IS from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (5.5, 480.0, and 780.0 pg/mL) was determined by comparing peak areas obtained from the plasma sample, and the standard solution spiked with the blank plasma residue. Recovery of more than 50 % was considered adequate to obtain the required sensitivity.

Stability (freeze - thaw, auto sampler, bench top, long term)

Low quality control and high quality control samples (n=6) were retrieved from the deep freezer after three freeze-thaw cycles according to the clinical protocol. Samples were stored at -30 °C in three cycles of 24, 36 and 48 h. In addition, the long-term stability of CZ in quality control samples was also evaluated by analysis after 105 days of storage at -30 °C. Autosampler stability was studied following 53 h storage period in the autosampler tray with control concentrations. Room temperature stability was studied for 24.5 h period with control concentrations. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15% respectively of their nominal concentrations**.**

Results and Discussion

Method optimization

For the optimization of LC-MS/MS method several parameters and mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Empagliflozin were obtained with Phenomenex Synergi 4.6µm(4.6×76mm)column and mobile phase containing a mixture of 5mM Ammonium Acetate buffer with 0.1% Formic Acid: Acetonitrile in the proportion of (50:40, v/v) was delivered at a flow rate of 0.900 mL/min by positive ion mode (API 4000) with injection volume of 20 µL and a run time of 3 min. Detection is performed by atmospheric pressure electro spray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z*451.52>71.32 for Empagliflozin and *m/z*267.3>226.8 for Nevirapine (Internal standard) were used for quantization was shown in Figure no.2&3. The retention time of Empagliflozin and Nevirapine (Internal standard) was found to be 1.50 min and 2.40 min respectively. A typical chromatogram of blank plasma, Empagliflozin and Nevirepin (Internal standard) is shown in Figure no. 4 & 5

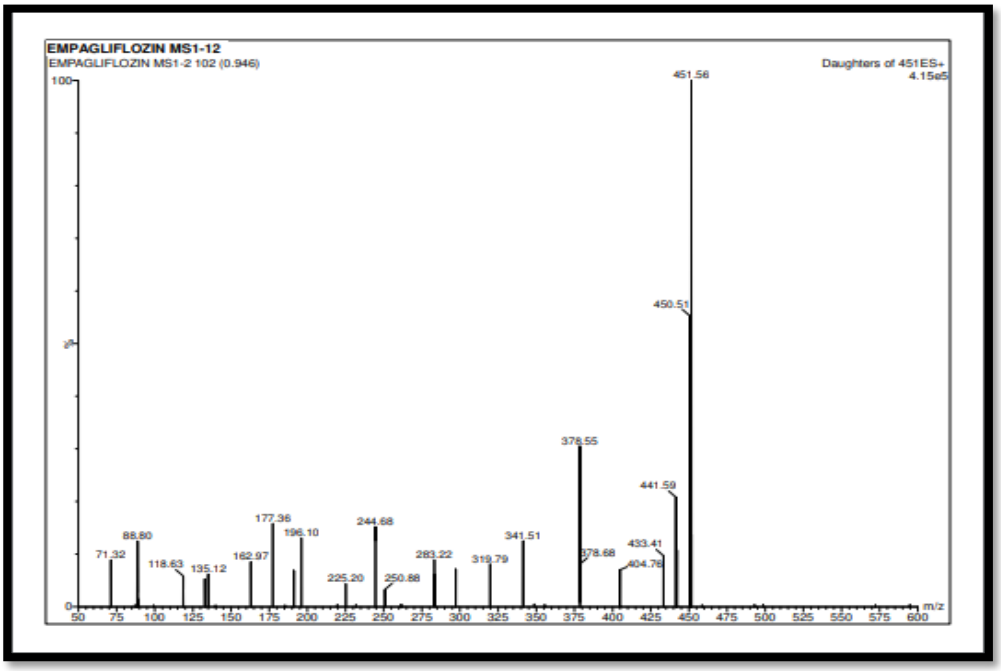

Fig 2. Mass spectra of Empagliflozin for precursor and product ion masses

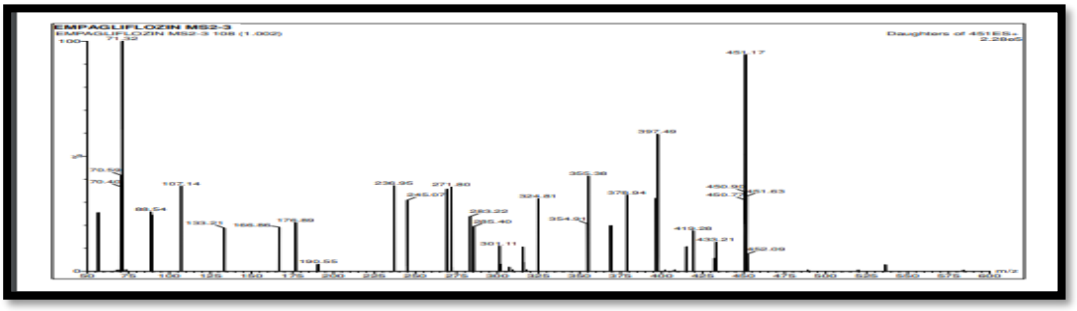

Fig 3. Mass spectra of IS for precursor and product ion masses

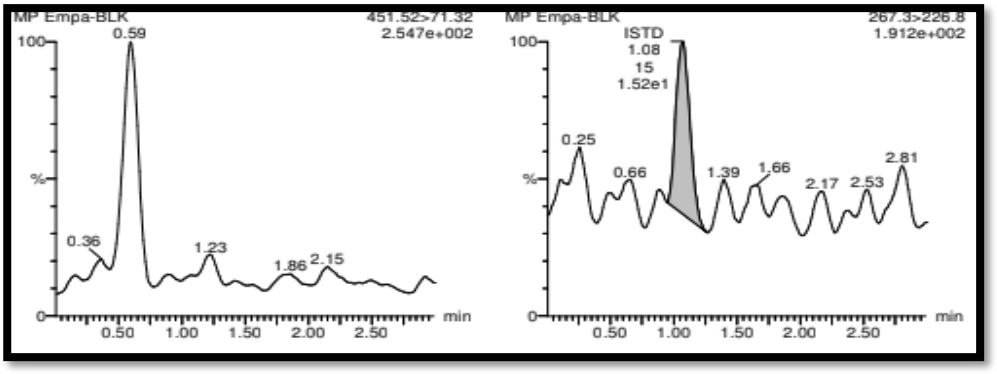

Fig 4. Blank chromatogram of Empagliflozin

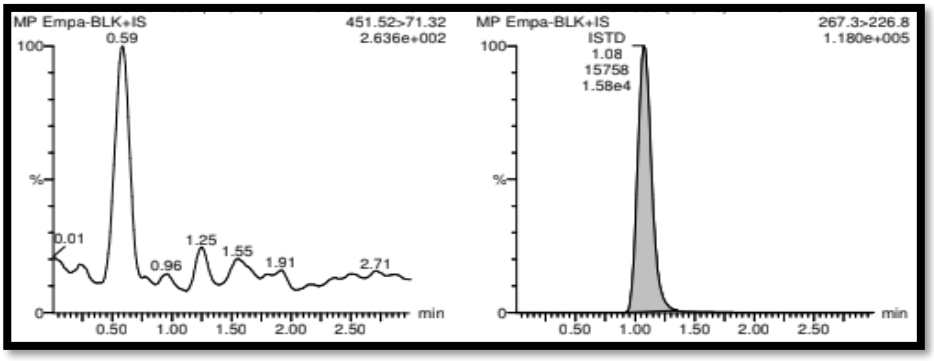

Fig 5. Blank chromatogram of Internal Standard

Matrix effect

No significant matrix effect was observed in all the eight batches including haemolysis and lipemic plasma for Empagliflozin at low (LQC) and high (HQC) concentrations. The precision and accuracy forEmpagliflozin at LQC concentration was found to be 0.05 % and 100.03 % respectively and at HQC concentration was found to be 0.01 % and 100.01 % respectively

Sensitivity

The lowest limit of reliable quantification (LLOQ) for Empagliflozin was set at the concentration of 2.0pg/mL. The precision and accuracy for Empagliflozin at this concentration was estimated.

Linearity

The linearity of Empagliflozin was assessed at six concentration levels in the range of2.0, 2.5, 16.5, 50.7, and 190.9 in plasma samples. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained.

Extraction recovery

Twenty-four blank matrix samples were processed and six sets of each blanks samples were reconstituted with the aqueous quality control dilutions at low, middle and high concentration without internal standard, which represents 100 % extraction of analyte(s) (non-extracted samples). Six blanks were reconstituted with the internal standard solution, which represents 100 % extraction of internal standard (Non-extracted sample). The non-extracted samples were injected. The recovery comparison samples of Empagliflozin were compared against extracted samples of LQC, MQC and HQC of PA Batch-I (Precision and accuracy). The recovery comparison samples of the internal standard were compared against the response of internal standards at MQC level.

Table 1
Calibration curve details from one batch of the validation section

Spiked plasma concentration (ng/ml)	The concentration measured ng/ml	SD	% CV	Accuracy %
2.0	2.1	0.03	0.4	98.9
2.5	2.6	0.08	0.08	99.8
16.5	16.3	0.13	0.02	99.9
50.7	50.6	0.10	0.06	101
190.9	190.71	0.11	0.04	99.9
487.2	487.4	0.21	0.04	100

Table 2
Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

	Within -run			Between –run		
Spiked plasma concentration ng/ml	Concentration measured (n=6) Mean ± SD	% CV	Accuracy	Concentration measured (n=6) Mean ± SD	%CV	Accuracy
1.00	1.443±1.886	1.3	98	1.024±0.11	0.11	98.01
5.5	5.544± 0.025	0.44	99.8	5.518±0.01	0.06	99.98
480.0	480.38±0.200	0.04	99.9	480.2±0.03	0.03	99.99
780.0	780.38±0.225	0.02	99.9	780.2±0.02	0.02	99.99

Recovery of the Empagliflozin and Nevirapine (Internal standard)

Recovery for Empagliflozin was found to be in the range of 92.14% to 96.19% and the mean recovery for Empagliflozin was 94.63 %. While for Nevirapine (Internal standard) the mean recovery was 91.94 %.

Room temperature stock solution stability

The stability was found to be 100.2 % for Empagliflozin with the precision ranged from 0.11% to 0.14 %. The stability was found to be 100.1 % for Nevirapine (Internal standard) with the precision ranged from 0.11 % to 0.35 %.

Bench top stability

Empagliflozin was found to be stable up to 6 hours as per the acceptance criteria. The percent mean nominal ranged from 100.02 % to 100.07 % and the precision ranged from 0.014 % to 0.078 %.

Freeze-thaw stability

Freeze-thaw stability of Empagliflozin is shown in Table. The percent nominal ranged from 99.98 % to 99.99 % and the precision ranged from 0.020 % to 0.121 % for four freeze-thaw cycles.

Table 3
Stability of samples

Spiked plasma concentration (ng/ml)	Short term stability (24.5 h) Concentration measured (n=6) Ng/ml (Mean ±SD)	(%cv)	Autosampler stability (53h) Concentration measured (n=6) Ng/ml (Mean ±SD)	(%cv)	Long-term stability (105 h) Concentration measured (n=6) Ng/ml (Mean ±SD)	(%cv)	Freeze thaw stability Cycle 3 (48h) Concentration measured (n=6) Ng/ml (Mean ±SD)	(%cv)
5.5	5.525 ±0.01	0.29	5.522±0.009	0.17	5.528±0.008	0.15	5.525±0.01	0.2
780.0	782.2±1.06	0.12	780.3±0.08	0.01	780.3±0.1	0.12	780.3±0.125	0.16

Conclusion

The present LC-MS/MS method for the estimation of Empagliflozin in human plasma by using Nevirapine as an internal standard was established and validated as per ICH guidelines. The developed and validated methods shown high degree of sensitivity, selectivity, reproducibility and high recovery, stability with less matrix effects.The chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method. It is highly specific, precise accurate, rugged and robust analytical procedure and allows the analysis of large number of samples in a short period of time. So, this method can be used for routine analysis.

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