ORIGINAL ARTICLE

Bioanalytical Method Development and Validation of Empagliflozin by LC–MS/MS Method and Quantitative Estimation of Drug Concentration in Human Plasma

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Abstract

Objective: The objective of this work is to develop rapid, selective, and sensitive liquid chromatography tandem–mass spectrometry (LC–MS/MS) method for the quantitative estimation of empagliflozin. Sample and standard solutions were prepared using methanol. **Methodology:** The chromatographic separation was achieved with X Bridge C18 column (75 mm × 4.6 mm, 3.5 μ) using a mobile phase composition of acetonitrile and 10 mM ammonium bicarbonate (70:30 V/V) at a flow rate of 0.8 mL/min with a run time of 2.40 min. The method showed good linearity in the range of 2–1000 ng/mL with correlation coefficient (r) of >0.9998. **Results:** The % CV of peak area ratio (analyte area/ISTD area) and % CV of retention times for analyte and ISTD were within the acceptance criteria. There was no significant carry over observed during this experiment. All the investigated human plasma lots were found to be free of significant interferences at the retention time of drug and ISTD. The intra- and inter-day precision values for empagliflozin comply with the acceptance criteria. The battery of stability studies, namely, bench-top, freeze-thaw, and long-term stability was performed. All the stability studies showing the % C.V. of area responses for the replicate injections should be within 15%. **Conclusion:** The developed method was very simple, precise, reliable, sensitive, and robustness. The retention time takes less time consumption and high sensitivity, the method applicable for routine analysis and bioanalysis.

Key words: Acetonitrile, empagliflozin, liquid chromatography tandem–mass spectrometry, methanol, stabilities

INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products and include all the procedures demonstrating particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, reliable, and reproducible for the intended use.[1] The recent studies show that sample throughput is an important part in bioanalytical method development involving an efficient preparation.[2] The analysis thus carried out must be verified for its alleged purpose and must be validated. An investigation should be performed during each step to determine whether the external environment, matrix, or procedural variables can affect the estimation of analyte in the matrix from the time of collection up to the time of analysis. Recent progress in methods development

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has been largely a result of improvements in analytical instrumentation.[3,4]

Empagliflozin is chemically known as (2S,3R,4R,5S,6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3-yloxy] phenyl} methyl) phenyl]-6-(hydroxy methyl) oxane-3,4,5-triol. It is a sodium glucose cotransporter-2 (SGLT-2) inhibitor. SGLT2 cotransporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucuronic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, resulting in increased glucose excretion. In addition, it contributes to reduced hyperglycemia, assists in weight loss, and reduces blood pressure. The chemical structure of empagliflozin is shown in Figure 1.

From the literature, high-performance liquid chromatography-ultraviolet, liquid chromatography tandem–mass spectrometry (LC–MS/MS), and ultra-performance liquid chromatography methods[5-8] have been reported for empagliflozin in various biological media. Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turnaround time during analysis.[9] At present, there is a need in the pharmaceutical environment to develop bioanalytical methods for the determination of empagliflozin in human plasma. For the estimation of the drugs present in the biological fluid, LC–MS/MS method is considered to be more suitable since this is a powerful and rugged method.[10] It is also extremely specific, linear, precise, accurate, sensitive, and rapid. The developed method could then be applied to clinical trials to obtain accurate pharmacokinetic parameters in human plasma.[11]

MATERIALS AND METHODS

Chemicals and instruments

Active pharmaceutical ingredients of empagliflozin and empagliflozin D_4 were obtained from Clearsynth Laboratories, Mumbai. LC–MS grade methanol and acetonitrile were provided by the Scharlau Chemicals Ltd., Ahmedabad. Methyl tertbutyl ether (TBME) and sodium hydroxide were purchased from Merck, Mumbai. Shimadzu LC was used. It was equipped with model UFLC XR and AB SCIEX make mass spectrometer equipped with API4500 Q-TRAP model which was used.

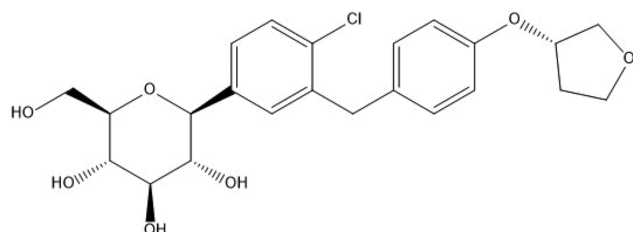

Figure 1: Chemical structure of empagliflozin

Preparation of empagliflozin standard solution

Accurately 5 mg of empagliflozin was weighed and transferred into 25 mL volumetric flask. It was dissolved in methanol, and the volume was made up to the mark and the resulting stock solution was stored at 2–8°C for further usage.[12]

Preparation of empagliflozin D_4 standard solution

Two milligrams of empagliflozin D_4 were weighed and transferred into 20 mL volumetric flask. It was dissolved in methanol, and the volume was made up to the mark and the resulting stock solution was stored at 2–8°C for further usage.[13]

Selectivity of concomitant drugs

The concomitant effect of drugs on the method was performed using standard (STD) and blank (BLK) by spiking concomitant drug solution separately for each concomitant drug, namely, ondansetron hydrochloride (225.979 ng/mL), caffeine (10226.883 ng/mL), paracetamol (23,822.328 ng/mL), ibuprofen (7995.071 ng/mL), hyoscine butylbromide (11.186 ng/mL), pantoprazole sodium (8005.898 ng/mL), nicotine Bi-L-(+)-tartrate (112.828 ng/mL), diclofenac sodium (2175.434 ng/mL), and pheniramine maleate (918.717 ng/mL)] and six samples equivalent to lower limit of quantification (LLOQ) and LLOQ quality control (QC) here using screened blank plasma. Concomitant drug stocks were prepared in methanol and further dilutions were prepared using methanol and stored.[14]

Preparation of mobile phase

Mixture of acetonitrile and 10 M ammonium bicarbonate buffer was prepared in the volume ratio of 70:30 and mixed well. The solution was stored at 25 ± 5°C and was used within 5 days from date of preparation.

Preparation of calibration curve (CC) standards

The CC stock dilutions were prepared in dilution solution as described in the table below.

Sample extraction

Six number of plasma samples were retrieved from the deep freezer, thawed water bath at room temperature, and vortexed to mix. Fifty microliters of empagliflozin D_4 internal standard dilution were taken in pre-labeled polypropylene tubes, except in standard blank samples wherein 50 μL of diluted methanol solution was taken. Two

hundred microliters of plasma samples were aliquoted into above polypropylene tubes vortexed to mix, then 50 µL of extraction buffer (0.2 N sodium hydroxide) was added and vortexed to mix. Two milliliters of methyl tertbutyl ether (TBME) were added to all the polypropylene tubes and were vortexed for 5 min at 2500 rpm.[15] All the polypropylene tubes were centrifuged at 4000 rpm, at 5°C for 5 min. 1.5 mL of supernatant was collected and transferred into pre-labeled RIA vial and was evaporated under nitrogen gas at 40°C till dryness. The dried residue was reconstituted with 0.5 mL of methanol or acetonitrile and vortexed to mix, then transferred to pre-labeled autosampler vials. The samples were injected to LC–MS/MS instrument for analysis.[16]

Bioanalytical method validation

System suitability

System suitability was performed to ensure that all analyzing parameters of the method such as reagents, samples, columns, instruments, and glass wares are suitable for the intended method. This was performed by injecting six subsequent injections of quality control samples (AQMQC) from a single vial.[17]

Carryover effect

Carryover is an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument. Carryover should be assessed and minimized during method development. During validation, carryover was assessed by analyzing blank samples after the calibration standard at the upper limit of quantification (ULOQ).

Matrix effect

A matrix effect is defined as alteration of analyte response due to interfering and unidentified component in the sample matrix. During method validation, it is necessary to evaluate the matrix effect between different independent sources/lots. Matrix factor was assessed using 10 different lots (six normal plasma, two hemolytic plasma, and two lipidemic plasma) of previously screened plasma lots. Blank samples in duplicate for each lot in each level were processed as per the respective method SOP.[18] Samples were spiked to achieve the concentration equivalent to high concentration quality control (HQC) and low concentration quality control samples (LOQ) which were injected. Unextracted samples concentration equivalent to HQC and LQC were also prepared and injected.

Linearity

The linearity of the method was determined using $1/x^2$ weighted least square regression analysis of standard plots associated with a 10-point standard curve. All the three CCs were analyzed and found to be linear for the concentration ranging from 2 to 1000 ng/mL.[19]

Sensitivity

Sensitivity was determined in terms of LLOQ QC, "Lower limit of Quantification Quality Control" which is the lowest concentration of analyte in a sample which can be quantified reliably with an acceptable accuracy and precision. Six LLOQ samples along with one set of CC standards and quality control samples were prepared and spiked with respective aqueous dilutions in blank matrix that has acceptable interfering area.[20,21] The LLOQ QC concentrations were back calculated using the (A and P batch) CC data.

Accuracy and precision

The accuracy and precision of the assay was evaluated by analyzing six replicates at different concentration levels corresponding to LLOQ QC, LQC, MQC, and HQC during the course of the method validation. The accuracy was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.[22]

Ruggedness

One accuracy and precision batch was processed using different sets of reagents by different analyst, analyzed using different column (same type), and performed on different instrument.

Reinjection reproducibility

Reinjection reproducibility was evaluated to determine if any analytical run could be reinjected in case of technical problem/instrument interruption. Six sets of LOQ, MQC, and HQC samples were prepared, then reinjected to the chromatographic system and analyzed for reproducibility of results.

Stability studies

Stability evaluations were carried out to ensure that every step taken during sample preparation, processing and analysis, as well as the storage conditions used do not affect the concentration of the analyte. All stability exercises involving biological matrix were freshly processed and analyzed along with CC standards and six sets of quality control samples (low-, middle-, and high-quality control samples) were prepared from freshly prepared stock solutions.[23]

Short-term stock solution stability for analyte and internal standard

Short-term stock solution stability for the analyte was determined after storage of stock solution in six replicates separately over a period of 7 h at room temperature. After 7 h,

dilutions were prepared from the stock at aqueous standard equivalent to ULOQ and LLOQ concentrations.

Freeze-thaw stability

Freeze-thaw stability was carried out to assess the stability of the analyte in biological fluids during repeated freezing and thawing cycles. After completion of freezing, six sets quality control samples from deep freezer were withdrawn and stored at room temperature. The samples were refreezing again. The quality control samples were frozen for 24 h for the first cycle, 12 h between each subsequent cycle of freeze and thaw. On the day of stability evaluation, after completion of freezing samples was withdrawn and stored at room temperature. CC standards and quality control samples were prepared from freshly prepared stock solutions. One set of CC standards and quality control samples was spiked with prepared dilutions in acceptable blank matrix.[24]

Bench top stability

The bench top stability of the spiked quality control samples was determined for a period of 6 h 27 min at room temperature.

Autosampler stability

Autosampler stability of the processed quality control samples was determined for a period for 2 days and 2 h by placing them in autosampler maintained at temperature of 10°C [Tables 1 and 2].

Long-term stability

Long-term stability was carried out to assess the test stability of analyte in biological fluids during its storage in deep freezer for a longer duration. From the freshly made stock solution, six sets of CC standards and quality control samples were prepared. Six sets of long-term stability quality control samples were withdrawn and stored at 70°C ± 10°C and −25°C ± 5°C, and freshly prepared stability samples were also stored under the same freezing condition.

Table 1: Preparation of calibration curve standards

Solution ID	Stock concentration (ng/mL)	Stock dilution concentration (ng/mL)	Stock dilution ID
EMPA-CC	204,937.480	50,086.720	AQ-STD-10
AQ-STD-10	50,086.720	42,573.712	AQ-STD-9
AQ-STD-9	42,573.712	30,048.526	AQ-STD-8
AQ-STD-8	30,048.526	15,024.263	AQ-STD-7
AQ-STD-7	15,024.263	7512.132	AQ-STD-6
AQ-STD-6	7512.132	3756.066	AQ-STD-5
AQ-STD-5	3756.066	1250.019	AQ-STD-4
AQ-STD-4	1250.019	500.008	AQ-STD-3
AQ-STD-3	500.008	200.003	AQ-STD-2
AQ-STD-2	200.003	100.002	AQ-STD-1

RESULTS AND DISCUSSION

System suitability showing the % CV of peak area ratio (analyte area/ISTD area) and % CV of retention times for analyte and ISTD were within the acceptance criteria [Figure 2]. The results are summarized in Table 3.

Table 2: Bioanalytical conditions

Chromatographic conditions and mass spectrometric parameters		
Column details	X Bridge C18 4.6 mm X 75 mm, 3.5 µm	
Mobile phase	Acetonitrile:mobile phase buffer (70:30 v/v)	
Flow rate	0.800 mL/min	
Column oven temperature	40°C	
Autosampler temperature	10°C	
Volume of injection	20 µl	
Detector	Mass detector	
Retention time of analyte	At about 1.09 min	
Retention time of ISTD	At about 1.08 min	
Run time	About 2.40 min	
Ion source	Turbo spray ionization	
Scan type	Multiple reaction monitoring (MRM)	
MRM transition	Empagliflozin: 449.200/371.000 (m/z) Empagliflozin D_4: 453.200/375.100 (m/z)	
Polarity	Negative	
Pooled matrix and expiry date	(lots used for pooled matrix preparation – LS/CLI/19/0246, LS/CLI/19/0368, LS/CLI/19/0369, LS/CLI/19/0370, LS/CLI/19/0371, and LS/CLI/19/0372)	July 13, 2020
Preparation of CCs and QCs/bulk spiking date	November 5, 2019	
Storage conditions	−70±10°C and −25±5°C	
Storage location and instrument	Deep freezer area and DF-001 (−70±10°C) and DF-004 (−25±5°C)	

There was no significant carryover observed during this experiment. Interference was not observed at the retention times of analyte and internal standard carryover was observed during the experiment [Figure 3]. The results are summarized in Table 4.

The % C.V. of unextracted aqueous comparison samples at LQC and HQC level for empagliflozin and empagliflozin D_4 was 8.24%, 8.19% and 6.68%, 6.74%, respectively. The matrix factor mean of empagliflozin for extracted LQC and HQC samples was found to be 1.024 and 1.025 [Figure 4]. The results and the acceptance criteria are summarized in Table 5.

The % accuracy of back calculated concentrations for LLOQ (STD-1) was found to be 98.40% and the LLOQ (STD-2 to STD-10) was ranged from 94.48% to 104.74%. The % C.V. of back calculated concentrations for LLOQ (STD-1) was found to be 0.56% and LLOQ (STD-2 to STD-10) was ranged from 0.27% to 3.14%. Representative chromatograms of CC are shown in Figure 5 which is obtained during the precision and accuracy batch. The results are summarized in Table 6, respectively.

The % C.V. and % accuracy at LLOQ QC level were found to be 5.06% and 97.85%, respectively. The mean S/N ratio was found to be 486.198. The results and the acceptance criteria are summarized in Table 7.

The % accuracy of back calculated concentration for LLOQ QC, LQC, MQC, and HQC samples was 97.90%, 91.63%, 94.18%, and 94.15%. The results and the acceptance criteria are summarized in Table 8.

The % C.V. of back calculated concentration for LLOQ QC, LQC, MQC, and HQC samples was 4.65%, 1.68%, 0.40%, and 0.37%. The results and the acceptance criteria are summarized in Table 8.

Ruggedness shown that the % C.V. of back calculated concentration for all the samples of LLOQ QC was found to be 7.83%. The % C.V. of back calculated concentrations for all quality control samples at LQC, MQC, and HQC concentration levels was 3.62%, 1.10%, and 0.76%. The % accuracy of back calculated concentration for all the samples of LLOQ QC was found to be 97.10%. The % accuracy of back calculated concentrations for all quality control samples at LQC, MQC, and HQC concentration levels was 87.69%, 93.58%, and 94.37%. The results of quality control samples are summarized in Table 9.

Stability data showing that the % C.V. of replicate injections of stability stock solution was found to be 1.94% and 0.78% for LLOQ and ULOQ concentrations levels, respectively, and the % C.V. of replicate injections of comparison stock

Table 3: System suitability

Sample ID	% CV		
	Analyte RT	ISTD RT	Area ratio
SYS01	1.25	1.52	1.402
SYS02	1.43	1.26	1.397
SYS03	1.51	1.91	1.457
SYS04	1.62	1.36	1.469
SYS05	1.47	1.28	1.376
SYS06	1.39	1.41	1.435

Table 4: Carryover effect for empagliflozin and empagliflozin D_4 (ISTD)

Sample IDs	Area at the RT of analyte	% interference at the RT of analyte	Area at the RT of internal standard	% interference at the RT of internal standard
COE-BLK-1	0	0.00	0	0.00
COE-BLK-2	0	0.00	0	0.00
COE-BLK-3	0	0.00	0	0.00
LLOQ sample				
Sample IDs		Area at the RT of analyte		
COE-STD-1		13,301		
LLOQ and DIQC Sample				
Sample IDs		Area at the RT of internal standard		
COE-STD-1		3,259,619		
COE-DIQC		2,777,919		
Mean area of internal standard		3,018,769.000		
LLOQ and ULOQ sample				
COE-STD-1		3,258,962		
COE-ULOQ		6,057,147		
Mean area of internal standard		4,658,054		

Table 5: Matrix effect of empagliflozin and empagliflozin D_4 (ISTD)

Unextracted aqueous comparison samples				
051119 matrix effect	**Area response in LQC sample**		**Area response in HQC sample**	
	Analyte	**Internal standard**	**Analyte area**	**Internal standard area**
	42,663	3,904,827	6,388,014	4,555,326
	44,043	4,058,018	6,189,474	4,428,566
	45,702	4,340,590	5,605,833	3,982,819
	41,495	3,962,539	5,527,511	3,958,920
	47,168	4,352,602	5,703,024	4,101,751
	51,918	4,838,201	5,434,441	3,864,008
Mean	45,498.167	4,242,796.167	5,808,049.500	4,148,565.000
S.D.	3747.671	347,286.072	387,827.410	279,448.124
% C.V.	8.24	8.19	6.68	6.74

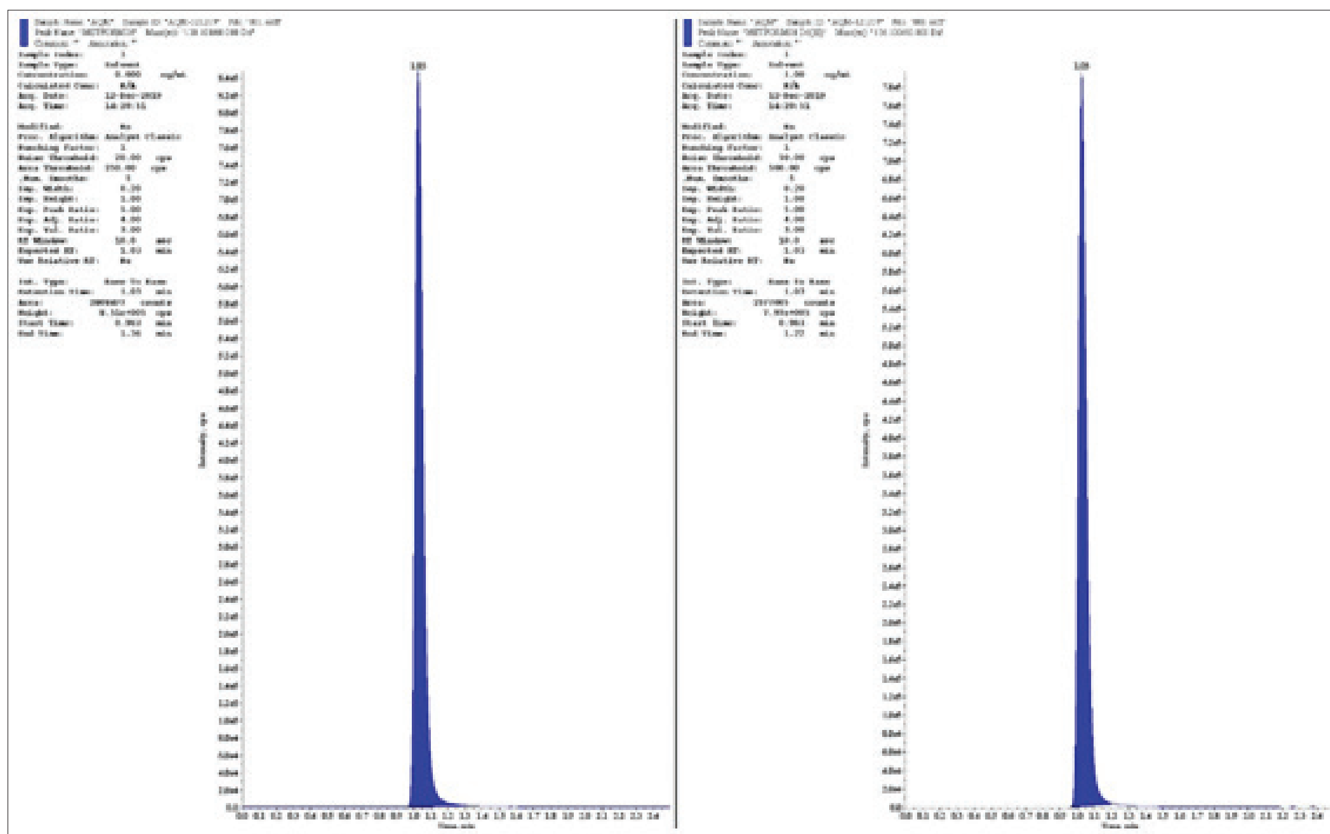

Figure 2: System suitability chromatogram of analyte and internal standard

solution was found to be 2.12% and 1.64% for LLOQ and ULOQ concentrations levels, respectively. The % comparison was found to be 102.93% and 97.53% for LLOQ and ULOQ, respectively, and the stability was proved for 7 h.

The results and the acceptance criteria are summarized in Tables 10 and 11. Seven freeze-thaw cycles were proved for freeze-thaw stability. The % C.V. was found to be 1.32% for LQC and 0.70% for HQC. The % nominal was found to be 93.39% for LQC and 94.36% for HQC. The results are summarized in Table 12a. Seven freeze-thaw cycles were proved for freeze-thaw stability. The % C.V. was found to be 1.10% for LQC and 0.18% for HQC. The % nominal was found to be 90.75% for LQC and 94.80% for HQC. The results are summarized in Table 12b.

The bench top stability was proved for 6 h 27 min at room temperature. The % C.V. was found to be 1.54% for LQC and 0.77% for HQC. The % nominal was found to be 96.58%

Table 6: Calibration curve data of accuracy and precision runs

Sample IDs	Nominal concentration (ng/mL)	Calculated concentrations (ng/mL)			Mean	S.D.	% C.V.	% nominal
		061119 AP1 and DI	061119 AP2 and REC	071119 AP3 and RIR				
STD-1	2.000	1.978	1.968	1.957	1.968	0.011	0.56	98.40
STD-2	4.000	4.008	4.048	4.072	4.043	0.032	0.79	101.08
STD-3	10.000	10.319	10.396	10.254	10.323	0.071	0.69	103.23
STD-4	25.000	25.886	25.553	27.115	26.185	0.823	3.14	104.74
STD-5	75.121	76.804	75.972	78.258	77.011	1.157	1.50	102.52
STD-6	150.243	152.751	153.392	153.543	153.229	0.421	0.27	101.99
STD-7	300.485	301.355	302.263	287.693	297.104	8.163	2.75	98.87
STD-8	600.971	592.157	589.252	603.941	595.117	7.779	1.31	99.03
STD-9	851.474	804.972	813.050	795.292	804.438	8.891	1.11	94.48
STD-10	1001.734	970.432	970.439	935.198	958.690	20.344	2.12	95.70
Slope		0.00181	0.0018	0.00182				
Intercept		0.000485	0.000531	0.000516				
Correlation coefficient (r)		0.9995	0.9996	0.9986				
Correlation coefficient (r^2)		0.9990	0.9992	0.9972				

Table 7: Sensitivity and S/N ratio of empagliflozin (LLOQ QC samples)

Accuracy and precision for sensitivity LLOQ QC samples			S/N ratio
Batch ID	LLOQ QC sample IDs	Calculated concentrations of LLOQ QC samples	
061119 AP1 and DI	AP1-LLOQ QC-1	1.708	84.446
	AP1-LLOQ QC-2	2.001	759.952
	AP1-LLOQ QC-3	2.075	456.030
	AP1-LLOQ QC-4	1.945	443.959
	AP1-LLOQ QC-5	2.000	155.873
	AP1-LLOQ QC-6	2.049	716.402
061119 AP2 and REC	AP2-LLOQ QC-1	1.917	657.106
	AP2-LLOQ QC-2	1.962	407.972
	AP2-LLOQ QC-3	1.983	1156.263
	AP2-LLOQ QC-4	1.963	508.460
	AP2-LLOQ QC-5	1.964	446.338
	AP2-LLOQ QC-6	1.934	364.769
071119 AP3 and RIR	AP3-LLOQ QC-1	1.924	333.987
	AP3-LLOQ QC-2	2.063	448.866
	AP3-LLOQ QC-3	1.728	213.271
	AP3-LLOQ QC-4	2.021	441.671
	AP3-LLOQ QC-5	2.026	518.964
	AP3-LLOQ QC-6	1.967	637.227
Mean		1.957	486.198
S.D.		0.099	NA
% C.V.		5.06	
Nominal concentration (μg/mL)		0.002	
% accuracy		97.85	
N (number of samples)		18	

Table 8: Quality control samples data for accuracy and precision runs

QC ID	LLOQ QC	LQC	MQC	HQC
Nominal conc. (ng/mL)	2.000	5.987	443.448	774.988
Nominal conc. lower range (ng/mL)	1.600	5.089	376.931	658.740
Nominal conc. upper range (ng/mL)	2.400	6.885	509.965	891.236
Batch ID and date	Back calculated conc. (ng/mL)			
061119 AP1 and DI	1.708	5.524	416.702	734.660
	2.001	5.409	418.998	724.852
	2.075	5.475	416.184	728.878
	1.945	5.574	416.091	731.295
	2.000	5.474	418.793	732.738
	2.049	5.587	415.730	730.103
N (number of samples)	6	6	6	6
Mean	1.963	5.507	417.083	730.421
S.D	0.133	0.068	1.439	3.394
%C.V	6.78	1.23	0.35	0.46
%accuracy	98.15	91.98	94.05	94.25
061119 AP2 and REC	1.917	5.468	419.849	729.278
	1.962	5.317	417.107	728.316
	1.983	5.339	416.778	728.431
	1.963	5.527	416.261	731.628
	1.964	5.557	420.628	726.215
	1.934	5.584	418.741	729.558
N (number of samples)	6	6	6	6
Mean	1.954	5.465	418.227	728.904
S.D	0.024	0.113	1.782	1.777
%C.V	1.23	2.07	0.43	0.24
%accuracy	97.70	91.28	94.31	94.05
Intraday accuracy and precision				
N (number of samples)	12	12	12	12
Mean	1.958	5.486	417.655	729.663
S.D	0.091	0.092	1.656	2.702
%C.V	4.65	1.68	0.40	0.37
%accuracy	97.90	91.63	94.18	94.15
AP3 and RIR 071119	1.924	5.595	406.458	729.688
	2.063	5.596	413.433	733.275
	1.728	5.806	409.336	721.607
	2.021	5.772	408.193	727.084
	2.026	5.868	398.234	722.818
	1.967	5.741	405.672	715.593
N (number of samples)	6	6	6	6
Mean	1.955	5.730	406.888	725.011
S.D	0.121	0.112	5.042	6.318
%C.V	6.19	1.95	1.24	0.87
%accuracy	97.75	95.71	91.76	93.55
Interday accuracy and precision				
N (number of samples)	18	18	18	18
Mean	1.957	5.567	414.066	728.112
S.D	0.099	0.152	6.044	4.643
%C.V	5.06	2.73	1.46	0.64
%accuracy	97.85	92.98	93.37	93.95

Table 9: Quality control samples data for ruggedness

Within batch accuracy and precision for ruggedness				
	Calculated concentrations (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
Nominal conc. (ng/mL)	2.000	5.987	443.448	774.988
Nominal conc. lower range (ng/mL)	1.600	5.089	376.931	658.740
Nominal conc. upper range (ng/mL)	2.400	6.885	509.965	891.236
061119 Ruggedness	Back calculated concentrations (ng/mL)			
	2.211	5.121	418.965	732.170
	1.832	5.024*	409.378	727.457
	1.873	5.091	409.580	727.548
	1.876	5.414	414.753	740.334
	2.033	5.435	417.861	725.871
	1.827	5.414	419.355	734.964
Mean	1.942	5.250	414.982	731.391
S.D.	0.152	0.190	4.559	5.551
% C.V.	7.83	3.62	1.10	0.76
% accuracy	97.10	87.69	93.58	94.37
N (number of samples)	6	6	6	6

Table 10: Stock solution stability of empagliflozin at room temperature

051119 RT STK and DIL stability	Analyte area response for LLOQ		Analyte area response for ULOQ	
	Stability stock solution	Comparison stock solution	Stability stock solution	Comparison stock solution
	14,950	14,090	6,194,744	6,220,336
	14,761	14,204	6,104,524	6,354,182
	14,331	13,878	6,152,940	6,312,346
	14,303	14,627	6,212,008	6,148,775
	14,271	13,905	6,136,528	6,439,621
	14,423	13,833	6,090,707	6,340,836
Mean	14,506.500	14,089.500	6,148,575.200	6,302,682.700
S.D.	281.463	298.860	48,184.440	103,365.260
% C.V.	1.94	2.12	0.78	1.64
Stock solution concentration	204,937.480	204,877.510	204,937.480	204,877.510
Stock solution IDs	EMPA-CC-041119-1 to 6	EMPA-CC-051119-1 to 6	EMPA-CC-041119-1 to 6	EMPA-CC-051119-1 to 6
% comparison	102.93		97.53	

for LQC and 96.83% for HQC. The results are summarized in Table 13.

The autosampler stability was proved for 2 days 2 h at 10°C. The % C.V. was found to be 1.31% for LQC and 0.26% for HQC. The % nominal was found to be 90.50% for LQC and 94.08% for HQC. The results are summarized in Table 14.

The long-term stability was proved for 25 days 17 h at −25 ± 5°C. The % C.V. was found to be 2.24% for LQC and 0.53% for HQC. The % nominal was found to be 92.42% for LQC and 93.71% for HQC. The results and the acceptance criteria are summarized in Table 15a.

The long-term stability was proved for 25 days 17 h at −70 ± 10°C. The % C.V. was found to be 1.67% for LQC and 0.48% for HQC. The % nominal was found to be 89.93% for LQC and 94.26% for HQC. The results and the acceptance criteria are summarized in Table 15b.

CONCLUSION

The LC–MS/MS method for specific quantitative measurement of empagliflozin in human plasma has been developed and validated. The developed method was very simple, precise, reliable, sensitive, and robustness. The retention time takes less time consumption. This method has high sensitivity. This

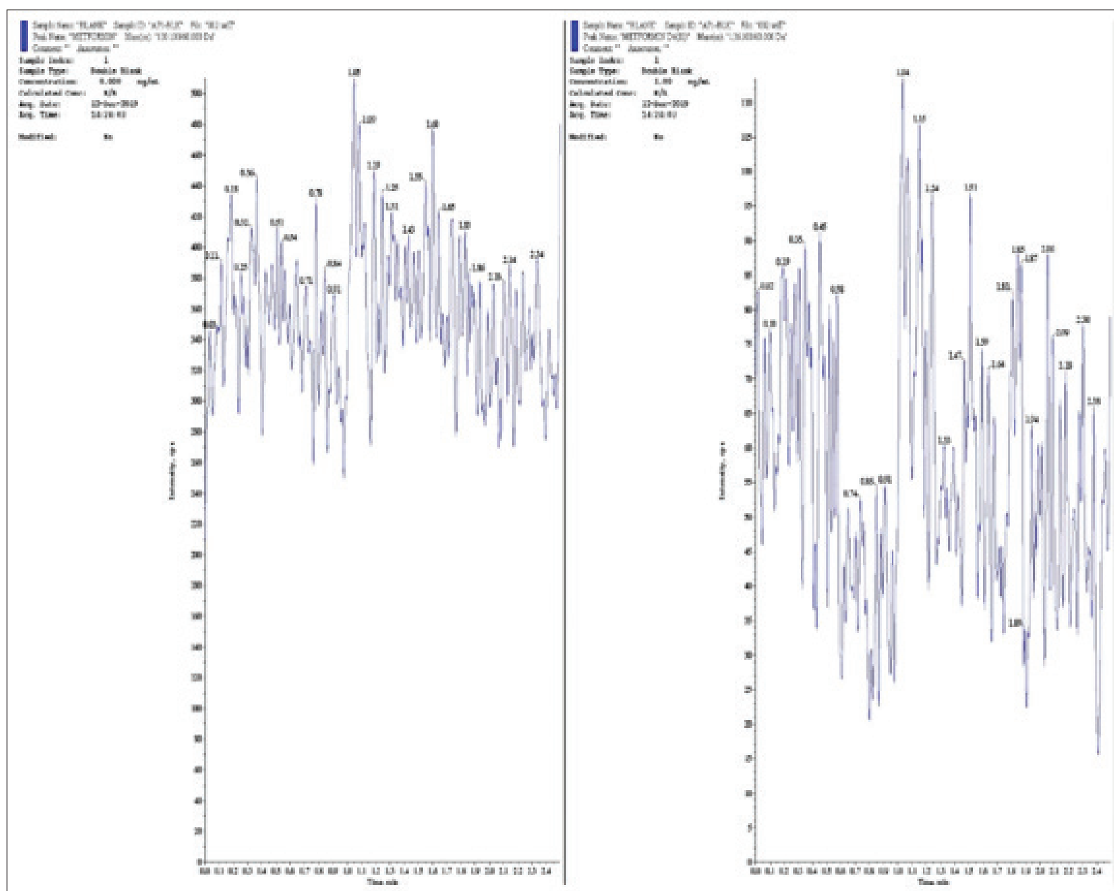

Figure 3: Chromatogram of blank matrix and internal standard

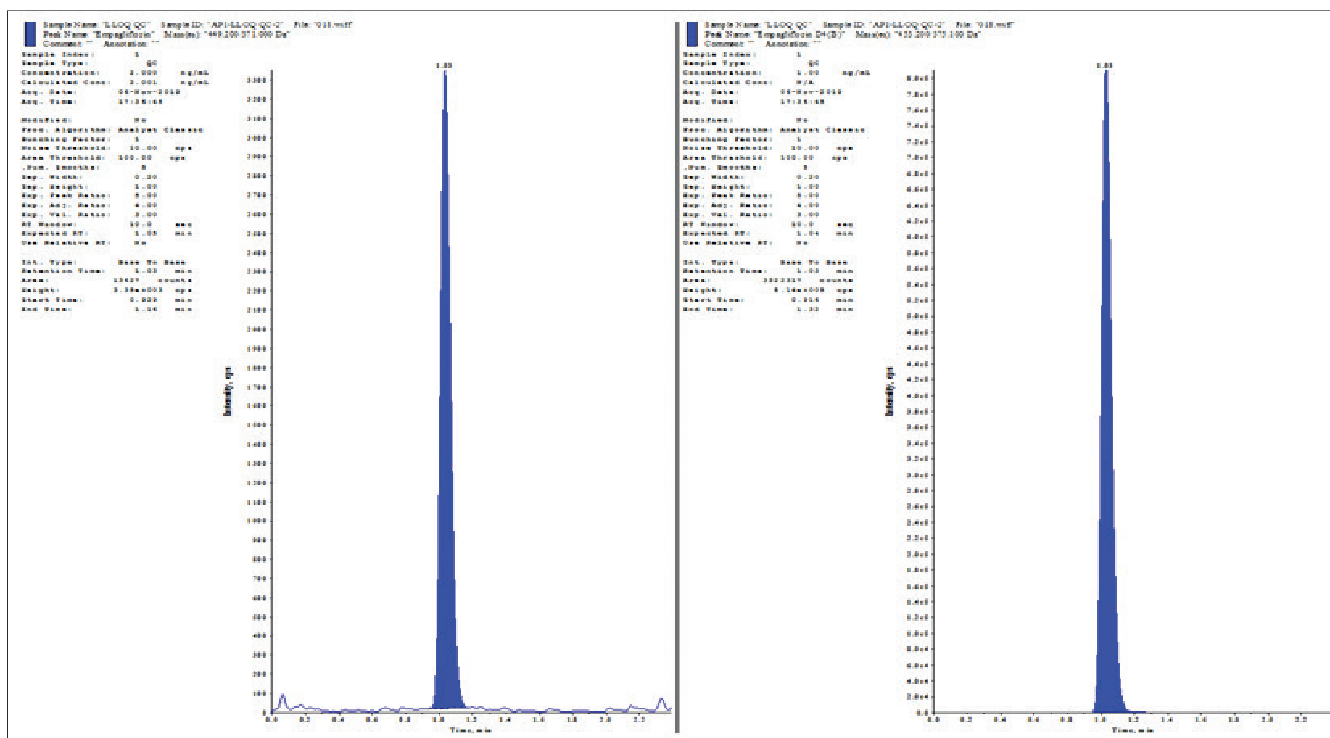

Figure 4: Chromatogram of lower limit of quantification standard

Table 11: Short-term stock dilution stability of empagliflozin at room temperature

051119 RT STK and DIL stability	Analyte area response for LLOQ		Analyte area response for ULOQ	
	Stability stock dilution	Comparison stock dilution	Stability stock dilution	Comparison stock dilution
	14,109	14,090	6,310,837	6,220,336
	13,934	14,204	6,260,036	6,354,182
	14,248	13,878	6,266,611	6,312,346
	14,781	14,627	6,268,108	6,148,775
	14,707	13,905	6,430,522	6,439,621
	13,881	13,833	6,237,595	6,340,836
Mean	14,276.667	14,089.500	6,295,618.200	6,302,682.700
S.D.	385.343	298.860	70,230.560	103,365.260
% C.V.	2.70	2.12	1.12	1.64
Stock dilution concentration	100.173	100.480	50,086.720	49,990.112
Stock dilution IDs	LLOQ-041119-1 to 6	COMP-LLOQ-051119-1 to 6	ULOQ-041119-1 to 6	COMP-ULOQ-051119-1 to 6
% comparison	101.64		99.70	

Table 12a: Freeze-thaw stability of empagliflozin at −25±5°C

(7th cycle)		
QC samples	Stability LQC	Stability HQC
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
111119 STAB FT and PE(RF)	Back calculated conc. (ng/mL)	
	5.668	721.329
	5.659	733.542
	5.480	730.866
	5.579	735.543
	5.536	733.670
	5.624	732.840
Mean	5.591	731.298
S.D.	0.074	5.111
% C.V.	1.32	0.70
% nominal	93.39	94.36
N (number of samples)	6	6

Table 12b: Freeze-thaw stability of empagliflozin at −70±10°C

(7th cycle)		
QC samples	Stability LQC	Stability HQC
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
111119 STAB FT and PE(RF)	Back calculated conc. (ng/mL)	
	5.508	734.406
	5.371	735.076
	5.480	734.392
	5.424	734.802
	5.357	732.635
	5.455	736.622
Mean	5.433	734.656
S.D.	0.060	1.287
% C.V.	1.10	0.18
% nominal	90.75	94.80
N (number of samples)	6	6

method is applicable for routine analysis and bioanalysis. This method has also applicable to the measurement of empagliflozin concentration in plasma samples and may be useful in biomonitoring, pharmacokinetic, and toxicokinetic studies of empagliflozin. The proposed method demonstrates good stability conditions for drug in biological matrix.

Table 13: Bench top stability of empagliflozin

Stability duration: 6 h27 min		
QC samples	**Stability LQC**	**Stability HQC**
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
071119 STAB BT, DE, PE(RT), and WBS	Back calculated conc. (ng/mL)	
	5.719	753.311
	5.715	755.207
	5.837	751.959
	5.694	748.483
	5.923	753.974
	5.802	739.591
Mean	5.782	750.421
S.D.	0.089	5.784
% C.V.	1.54	0.77
% nominal	96.58	96.83
N (number of samples)	6	6

Table 14: Autosampler stability of empagliflozin

Stability duration: 02 days 2 h		
QC samples	**Stability LQC**	**Stability HQC**
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
081119 STAB ASS and LTSR1	Back calculated conc. (ng/mL)	
	5.401	726.737
	5.297	727.408
	5.387	731.261
	5.478	731.208
	5.460	729.021
	5.482	728.955
Mean	5.418	729.098
S.D.	0.071	1.875
% C.V.	1.31	0.26
% nominal	90.50	94.08
N (number of samples)	6	6

Table 15a: Long-term stability of empagliflozin at −25±5°C

Stability duration: 25 days 17 h		
QC samples	**Stability LQC**	**Stability HQC**
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
081119 STAB ASS and LTSR1	Back calculated conc. (ng/mL)	
	5.616	725.440
	5.689	721.480
	5.611	732.809
	5.476	727.380
	5.443	726.479
	5.364	723.650
Mean	5.533	726.206
S.D.	0.124	3.860
% C.V.	2.24	0.53
% nominal	92.42	93.71
N (number of samples)	6	6

Table 15b: Long-term stability of empagliflozin at −70±10°C

Stability duration: 25 days 17 h		
QC samples	**Stability LQC**	**Stability HQC**
Nominal conc. (ng/mL)	5.987	774.988
Nominal conc. lower range (ng/mL)	5.089	658.740
Nominal conc. upper range (ng/mL)	6.885	891.236
081119 STAB ASS and LTSR1	Back calculated conc. (ng/mL)	
	5.438	731.355
	5.402	730.378
	5.366	726.022
	5.327	736.658
	5.516	728.698
	5.255	729.761
Mean	5.384	730.479
S.D.	0.090	3.536
% C.V.	1.67	0.48
% nominal	89.93	94.26
N (number of samples)	6	6

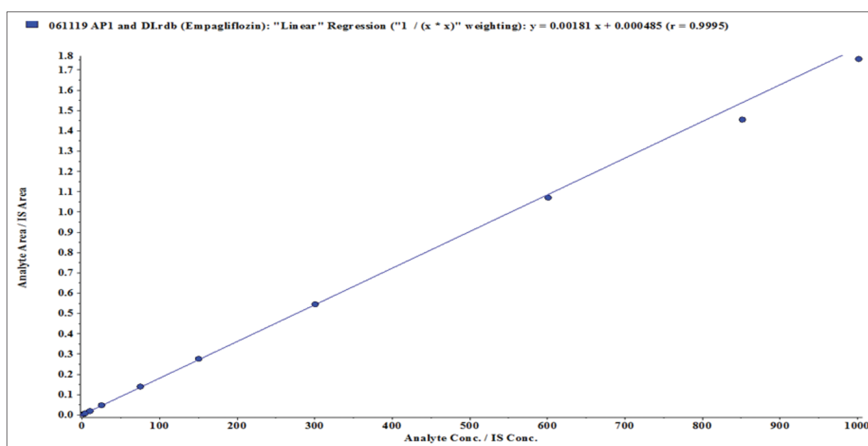

Figure 5: Calibration curve for empagliflozin

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