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A SIMPLE, SENSITIVE AND FAST SINGLE STEP EXTRACTION METHOD FOR DETERMINATION OF EMPAGLIFLOZIN IN HUMAN PLASMA USING LC-MS/MS

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ABSTRACT: A simple, sensitive, and fast single-step extraction method for estimating empagliflozin in human plasma using LC-MS/MS was developed and validated for pharmacokinetics evaluations. Plasma samples were basified before solid-phase extraction on SOLA (30 mg / 1 mL cartridges). Separations were carried out on a normal reverse phase C18 column (Hypersil BDS 100 × 4.6, 5µ mm column) for 3.5 minutes at a flow rate of 0.6 mL/min. Ten µL of the SPE eluent is directly injected onto LC-MS/MS to quantify the analyte from 1.563-800.000 ng/mL using a single SRM transition (m/z: 449.140→371.100) in negative ion mode. During method validation, selectivity, matrix effect, recovery, carry-over effect, stability studies, inter-day, and intra-day precision and accuracy experiments were conducted per USFDA guidelines. Method validation data has successfully met the acceptance criteria making it suitable for use in routine bio-analytical laboratories. The scope of this assay can be extended to cover the requirement of preclinical, toxicology, and PK/PD studies.

INTRODUCTION: Empagliflozin is indicated as an adjunct to diet and exercise to improve glycemic control, assist in weight loss and reduce blood pressure in adult patients with type 2 diabetes. Empagliflozin inhibits the sodium-glucose co-transporter 2 which is responsible for the reabsorption of glucose from the glomerular filtrate in the kidneys resulting in glucuretic effect [1-5]. Based on the pharmacokinetic study data, the analytical method required for analysis of empagliflozin in human plasma must be sensitive to detect concentrations as low as 1.5 ng/mL.

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Moreover, the linear response relationship till the upper limit of quantification determines the applicability of the analytical method for multiple doses of the drug and makes the method preferable.

Few liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6-13] and diode array detectors (DAD/PDA) [14, 15] based bio-analytical methods were reportedly using ultra-performance liquid chromatography (UPLC) systems for estimation of empagliflozin in human plasma. In this method, we present a simple, sensitive, high-throughput, and robust method for the determination of empagliflozin in human plasma using the HPLC-MS/MS method in negative ion mode. The current study employs a simple and single-step extraction procedure with sample volumes as low as 200 µL, and the solid phase eluent is directly injected onto LC-MS/MS.

Run time was shorter under normal HPLC conditions with the ability to analyze over 400 samples per day.

MATERIALS: Methanol (gradient grade), acetonitrile (gradient grade), ammonium bicarbonate (LR grade), and ammonium acetate (LR grade) were purchased from Merck. Water (LCMS grade) was used in-house from Milli Q system. Empagliflozin and empagliflozin D_4 were procured from Vivan life sciences. Blank plasma was purchased from the blood bank. The chemical structure of empagliflozin is presented below in **Fig. 1**.

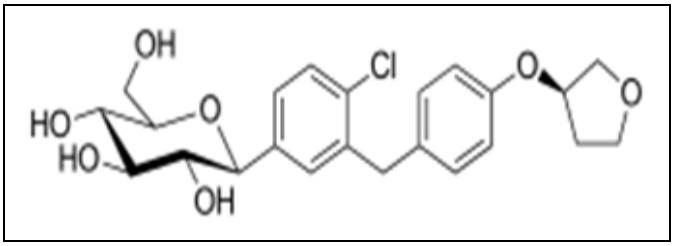


FIG. 1: STRUCTURE OF EMPAGLIFLOZIN

METHODS:

Instrumentation and Analytical Conditions: Ultimate 3000 HPLC system interfaced with a TSQ Endura Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc) was used for analysis. As empagliflozin is basic in nature, a heated electro-spray-ionization source was operated in the negative ion mode. Moreover, empagliflozin is moderately polar and lipophilic. Therefore, a reverse-phase LC column (Hypersil BDS C18 column-Dimensions: 100 × 4.6 mm, 5 µm) was used employed to resolve the analyte at oven temperature of 40 ºC. Acetonitrile: methanol: 10 mM ammonium bicarbonate in water (85:10:5 v/v/v) was used as a mobile phase. The retention time of both empagliflozin and empagliflozin D_4 was found to be the same at 1.8 min with a total run time of 3.5 minutes.

Mass spectrometry analysis was performed with the following optimized parameters: Sheath gas 50 (arb), auxiliary gas pressure 30 (arb), capillary temperature 300 °C, Q2 gas pressure 1.2 m Torr, ion spray voltage 3000 V, and vaporizer temperature 30 ºC. SRM (Selected reaction monitoring) transitions for quantification were m/z 449.140→371.100 for empagliflozin and 453.170 → 375.15 for empagliflozin D_4.

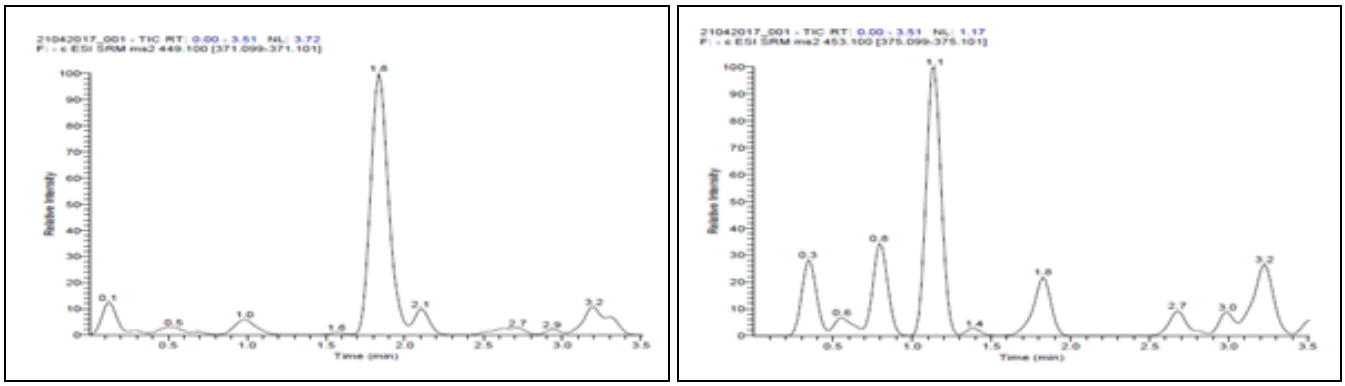
Preparation of Standard Solutions and Quality Control Samples: Standard solutions of empagliflozin (100 µg/mL) and empagliflozin D_4 (100 µg/mL) were prepared in methanol. Intermediate stock solutions of both analyte and internal standard (20µg/mL) were prepared in diluent (50% methanol in water) along with internal standard dilution (1µg/mL). Ten level calibrators and four-level controls were prepared in human plasma containing sodium heparin as anticoagulant from 1.563-800.000 ng/mL and 1.000-200.000 ng/mL, respectively.

Sample Preparation: Two hundred microliters of pre-spiked plasma samples were dispensed into microcentrifuge tubes. 30 µL of internal standard dilution (1000.000 pg/mL of empagliflozin D4) was dispended into all non-zero samples. Thirty microliters of diluent (50% methanol) were added to blank samples. Five hundred microliters of 2 mM ammonium acetate in water were used for pre-treatment to unionize the analyte and facilitate the reverse phase interactions during solid-phase extraction. Samples were vortexed to mix, and solid-phase extraction cartridges (SOLA-30 mg, 1mL) were conditioned and equilibrated with 1 mL methanol followed by 1 mL water (to improve water wettability and easily allow plasma samples to diffuse through the sorbent). Seven hundred and thirty microliters of pre-treated plasma sample were dispensed into the cartridges. Empagliflozin and internal standard were retained on the sorbent while the plasma components pass through the cartridges to waste due to gravity. To evaluate the retention mechanism of the analyte with the sorbent, solvents of different elution strength were verified in comparison with the physicochemical properties of empagliflozin, mainly solubility, polarity, and pH. This study has helped in eliminating other interfering compounds and separate the cleaner extracts of empagliflozin. Optimal clean-up of the samples was achieved with two wash solutions. Cartridges were initially cleaned with 900 µL water (2 times) followed by 900 µL 30% methanol in water. After drying the cartridges with nitrogen gas for 2 min, the analyte was extracted with 350 µL of acetonitrile: methanol: 2 mM ammonium acetate in water (70:20:10 v/v/v). The eluent was transferred to the HPLC vials, and 10µL was injected for LC-MS/MS analysis.

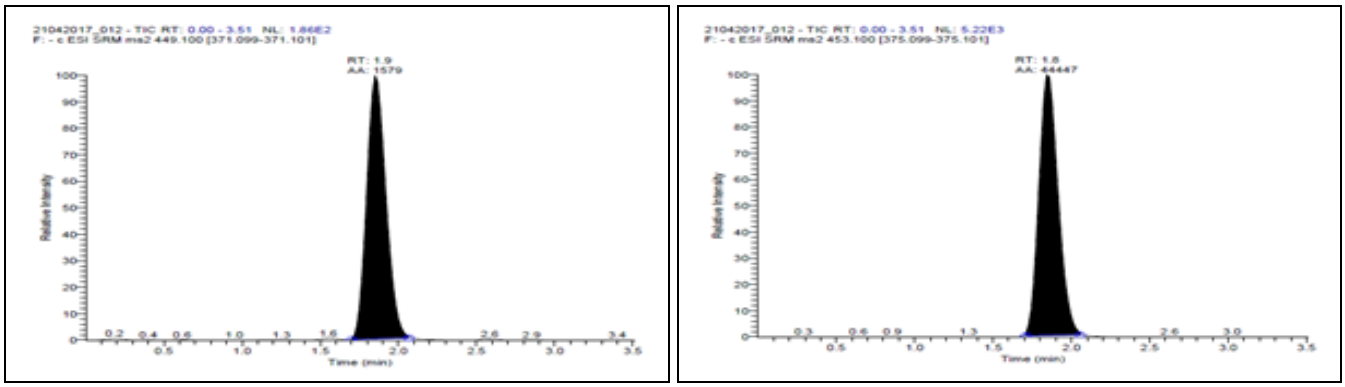
RESULTS:

Method Validation: Selectivity, linearity, precision, accuracy, recovery, and stability experiments were performed as per USFDA guidelines.

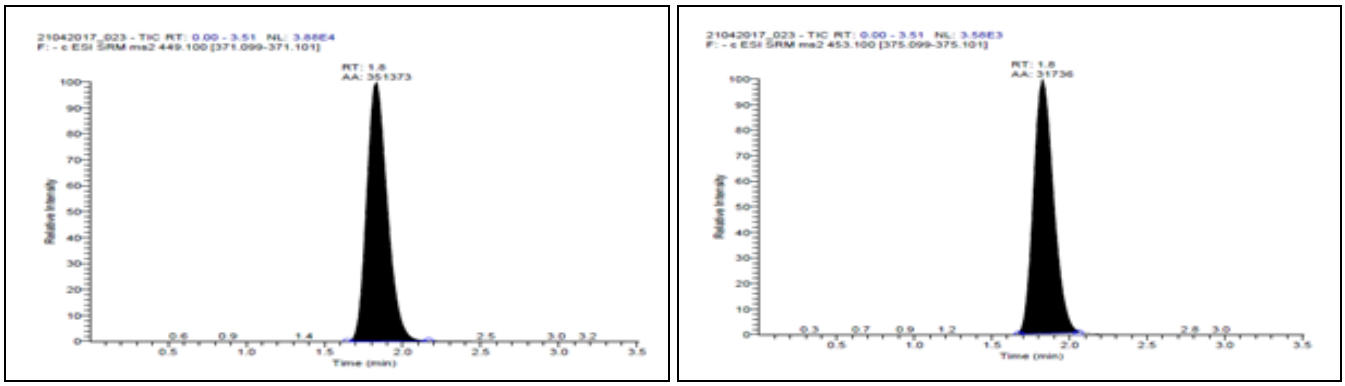
Representative chromatograms and a calibration curve of empagliflozin are presented in **Fig. 2** and **3**.



Extracted Blank



Extracted Lower limit of Quantification (LLOQ)



Extracted upper limit of quantification (ULOQ)

FIG. 2: REPRESENTATIVE CHROMATOGRAMS OF EMPAGLIFLOZIN

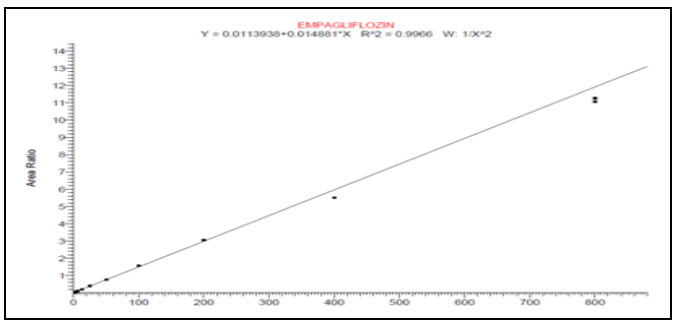


FIG. 3: CALIBRATION CURVE OF EMPAGLIFLOZIN IN HUMAN PLASMA FROM 1.563-800.000 ng/ml

Precision and accuracy (P&A) batches (includes ruggedness and stability PA batch) were analysed with the calibration cure ranging from 1.563-800.000 ng/mL. A straight-line equation (y=mx+c) with $1/x^2$ weighing factor has been used to quantify the back-calculated concentration of the calibrators and the coefficient of determination (r^2) was greater than 0.994 in all 5 PA batches. Summary of back-calculated concentrations and calibration curve parameters were presented below in **Table 1-2**.

TABLE 1: PRECISION AND ACCURACY

Standard name	Nominal concentration (ng/ml)	P&A-01	P&A-02	P&A-03	P&A-04	P&A-05	Mean ±SD	% CV	% Nominal
CS-1	1.6	1.6	1.6	1.6	1.6	1.7	1.63±0.02	1.36	104.07
CS-2	3.1	3.1	3.0	3.1	3.2	3.1	3.08±0.06	2.03	98.68
CS-3	6.3	6.3	6.2	6.3	6.2	6.2	6.23±0.05	0.77	99.69
CS-4	12.5	13.2	13.0	13.2	13.1	13.3	13.16±0.11	0.85	105.26
CS-5	25.0	26.4	25.3	26.5	26.0	26.4	26.13±0.51	1.94	104.52
CS-6	50.0	51.4	50.2	51.7	51.0	50.9	51.03±0.55	1.09	102.07
CS-7	100.0	105.2	104.1	105.1	105.0	104.1	104.69±0.58	0.55	104.69
CS-8	200.0	205.3	209.8	205.7	204.3	205.7	206.14±2.13	1.03	103.07
CS-9	400.0	370.1	381.4	369.6	370.3	369.3	372.12±5.21	1.4	93.03
CS-10	800.0	756.6	780.6	749.4	781.3	766.0	766.77±14.20	1.85	95.85

Mean statistical data are expressed as mean ± SD [n=5].

TABLE 2: CALIBRATION CURVE PARAMETERS SUMMARY

Result Table ID	Slope	Y-Intercept	Regression Coefficient [r]
P&A-01	0.0114	0.01	0.997
P&A-02	0.0096	0.14	0.998
P&A-03	0.0116	0.01	0.996
P&A-04	0.0161	0.01	0.994
P&A-05	0.0110	0.01	0.998

Specificity and selectivity of the method was assessed in 6 different lots of human plasma containing sodium heparin as an anticoagulant. Haemolyzed and lipidemic (each lot) were also used for the evaluation of selectivity of empagliflozin. % interference in blank was found to be less than 9% in all lots when compared against LLOQ area of empagliflozin. Results were presented below in **Table 3**.

TABLE 3: SELECTIVITY

	Empagliflozin			Empagliflozin D4		
Matrix Lot no	Area in Blank Matrix at analyte RT	Area of LLOQ	%Interference at retention time of Empagliflozin	Area in Blank Matrix at IS RT	Area of LLOQ	% Interference at retention time of Empagliflozin D4
LOT 1	95	1400	6.79	200	43590	0.46
LOT 2	83	1561	5.32	160	47967	0.33
LOT 3	75	1589	4.72	240	44810	0.54
LOT 4	106	1345	7.88	208	45006	0.46
LOT 5	35	1459	2.40	196	45072	0.43
LOT 6	51	1673	3.05	224	47418	0.47
LOT 7 (HEMOLYZED)	111	1532	7.25	300	43639	0.69
LOT 8 (LIPIDEMIC)	130	1472	8.83	252	44337	0.57

Intra-day precision and accuracy was evaluated in 6 replicates of control samples at LLOQ QC, LQC, MQC and HQC over one PA batch was found to be between 1.14-3.39% and 101.18-103.79 respectively. Intra-day precision and accuracy results were presented in **Table 4**.

TABLE 4: INTRA-DAY PRECISION AND ACCURACY

QC concentration	LLOQ QC	LQC	MQC	HQC
	1.563	6.250	50.000	200.000
Mean ± SD (n=6)	1.62±0.05	6.40±0.21	50.59±0.98	204.47±2.34
% CV	3.39	3.22	1.94	1.14
% Nominal	103.79	102.38	101.18	102.24

Mean statistical data are expressed as mean ± SD [n=6]

TABLE 5: INTER-DAY PRECISION AND ACCURACY

QC concentration	LLOQ QC	LQC	MQC	HQC
	1.563	6.250	50.000	200.000
Mean ± SD (n=30)	1.58±0.14	6.34±0.30	52.79±2.17	207.34±2.97
% CV	8.72	4.66	4.11	1.43
% Nominal	100.77	101.41	105.57	103.67

Mean statistical data are expressed as mean ± SD [n=30]

Inter-day precision and accuracy experiments were evaluated in 5 batches at the same levels mentioned above and the results were found to be between 1.43-8.72% and 100.77-105.57, respectively. Results of Inter-day precision and accuracy were tabulated in **Table 5**.

Matrix effect was studied for both empagliflozin and empagliflozin D_4 in eight lots of plasma (6 normal, 1 haemolyzed, and 1 lipidemic plasma). IS normalized matrix factor was calculated as a ratio of response ratio of post extracted spiked sample upon aqueous sample at both HQC and LQC concentration levels, and mean IS normalized matrix factor was found to be 1.00 and 0.95, respectively. Results of IS normalized matrix effect experiment were provided below in **Table 6**.

TABLE 6: MATRIX EFFECT EXPERIMENT

QC	Response ratio of matrix effect sample	Response ratio of aqueous standard	Matrix factor	QC	Response ratio of matrix effect sample	Response ratio of aqueous standard	Matrix factor
LQC	0.990	0.995	0.99	HQC	3.543	3.214	1.10
	0.868	0.935	0.93		3.221	3.376	0.95
	0.921	0.829	1.11		3.167	3.255	0.97
	0.835	0.944	0.88		3.084	3.192	0.97
	0.879	0.932	0.94		3.065	3.213	0.95
	0.888	0.913	0.97		3.158	3.735	0.85
	0.942	0.826	1.14		3.024	3.119	0.97
	0.961	0.945	1.02		3.117	3.616	0.86
Mean IS normalized matrix factor of LQC [mean ± SD (n=8)]			1.00±0.09	Mean IS normalized matrix factor of HQC [mean ± SD (n=8)]			0.95±0.08
% CV			8.86	% CV			8.22

The average recovery of empagliflozin was obtained by calculating the response ratio of extracted and aqueous samples at LQC, MQC, HQC levels and was found to be 93.73% and 102.16% for empagliflozin and empagliflozin D_4, respectively. Results of the recovery experiment were presented in **Table 7**.

TABLE 7: RECOVERY OF EMPAGLIFLOZIN

QC	Response of extracted sample	Response of Unextracted sample	% Recovery
LQC (n=6)	4422	4652	95.30
MQC (n=6)	29212	31891	91.60
HQC (n=6)	105037	111572	94.28
	Mean recovery (mean ± SD)		93.73±1.91
	% CV		2.04

Mean statistical data are expressed as mean ± SD [n=6x3]

TABLE 8: STABILITY EXPERIMENTS IN BIOLOGICAL MATRIX

Comparison QC details	Mean ± SD (n=6)	% CV	% Nominal
Freshly spiked LQC	6.40±0.0.21	3.28	102.46
Freshly spiked HQC	211.14±4.07	1.93	105.57
Stability QC details	**Mean ± SD (n=6)**	**% CV**	**% Nominal**
Autosampler stability-LQC (24 hrs)	6.44±0..28	4.28	103.05
Autosampler stability-HQC (24 hrs)	205.24±1.72	0.84	102.62
FT 5th Cycle LQC (-50 °C)	7.02±0.09	1.21	112.3
FT 5th Cycle HQC (-50 °C)	209.68±1.75	0.84	104.84
FT 5th Cycle LQC (-20 °C)	6.90±0.17	2.42	110.39
FT 5th Cycle HQC (-20 °C)	207.09±2.31	1.11	103.54
Wet extract stability LQC (40 hrs)	6.19±0.19	3.01	99.07
Wet extract stability HQC (40 hrs)	210.27±0.78	0.37	105.13
Bench top stability (LQC) (8 hrs)	6.49±0.20	3.13	103.91
Bench top stability (HQC) (8 hrs)	208.26±3.71	1.78	104.13
Long term matrix stability (LQC) (50 days)	6.18±0.15	2.51	98.96
Long term matrix stability (HQC) (50 days)	209.88±0.92	0.44	104.94

Stability experiments in matrix were conducted for bench-top, freeze-thaw (at -50 °C and at -20 °C), autosampler, wet extract, and long-term storage (at -50°C). Stock solution stability, reinjection reproducibility, ruggedness and dilution integrity were also performed during method validation. Results of these experiments met the acceptance criteria and were provided in **Table 8**.

DISCUSSION: Validation parameters and acceptance criteria of the results are mentioned below in **Table 9**.

TABLE 9: VALIDATION PARAMETERS AND THEIR ACCEPTANCE CRITERIA

S. no.	Parameter	Acceptance criteria
1	Linearity (includes ruggedness and stability)	Minimum 5-point standards are required for building a calibration curve Two consecutive standards should not fail First and last calibration standard should not fail At least 75 % of the calibration curve standards should be with the acceptable limits for accuracy and precision % Accuracy and precision should be within 85-115% for all standards except LLOQ. % Accuracy and precision should be within 80-120% for LLOQ
2	PA [(inter-day and intra-day) and (stability studies)]	% Accuracy and precision should be within 85-115% for all QCs except LOQ QC. % Accuracy and precision should be within 80-120% for LOQ QC. At least 67% of the quality control samples should be within specified criteria for precision and accuracy At least 50% quality control samples should meet the criteria specified for accuracy and precision
3	Specificity and selectivity	% interference at the retention time of the analyte in the blank sample should not be more than 20% of the peak area of analyte % interference at the retention time of the internal standard in the blank sample should not be more than 5% of the peak area of the internal standard
4	Matrix effect	Mean matrix factor and is normalized matrix factor should be between 0.85-1.15
5	Recovery	No as such criteria defined for % recovery. The precision obtained for mean and global recovery should be with in±15%
6	Dilution integrity (DI)	Precision and accuracy of the DI QCs should be within 85-115 %
7	Limit of detection (LOD)	Precision and accuracy of the sensitivity samples should be within 85-115%

Validation data was assessed as per the criteria mentioned above, and the results of all these parameters have met the acceptance criteria.

CONCLUSION: This newly developed bio-analytical method employs a relatively simple and fast extraction method as additional processing steps like centrifugation and evaporation are not required. The developed method was successfully validated as per USFDA guidelines, and it is suitable for the determination of empagliflozin in human plasma using LC-MS/MS for pharmaco-kinetic evaluations.

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CONFLICTS OF INTEREST: There is no conflict of interest to declare.

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