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Original Article

Development and Validation of a Bioanalytical Method to Determine Empagliflozin in Human Plasma Using UPLC-MS/MS

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ABSTRACT

Aim: The study we are conducting aims to establish a validated method for measuring empagliflozin in plasma using UPLC MS/MS. **Materials and Methods:** Sodium-Glucose co Transporter inhibitors (SGLTi), a novel class of diabetes medication. Non-insulin dependent diabetes and adult-onset diabetes were the prior names for type 2 diabetes. Empagliflozin was the first SGLT2-inhibitor a drug used that reduced the risk of cardiovascular risk those with type 2 diabetes and pre-existing cardiovascular disease. The empagliflozin D4 as Internal Solution (IS). Liquid-liquid extraction was used to achieve separation on Synergi 2.5µ Fusion-Reverse phase 100A (100 mm×2.0 mm), 2.5 µm column. **Mixture of Methanol:** As a mobile phase, 0.2% formic acid in a 75:25 volumetric ratio is utilised. The flow rate is set at 0.3µl/min. The calibration curve is linear and plotted. **Results:** The developed method is accurate, precise, sensitive method for determination of empagliflozin from human plasma solution. Ethyl acetate and water in HPLC grade are preferred solvent for empagliflozin. Performing LLE extraction with centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as non-polar solvent and water is used as polar solvent for liquid-liquid extraction. LLE is a low cost extraction process compare to solid liquid extraction. The reported method is suitable for bioequivalence and pharmacokinetic studies.

Keywords: Type 2 diabetes (T2D), Empagliflozin, Human Plasma, UPLC-MS/MS.

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INTRODUCTION

The micro- and macrovascular systems are both impacted by the chronic condition known as diabetes mellitus.[1-4] T2DM, formerly 6.4% of people worldwide already have adult-onset diabetes that is not dependent on insulin, generally known as the disease. and is projected to reach 7.7% by 2030–2022. Patients with type 2 diabetes are most commonly unwell and suffer from cardiovascular disease.[5-7] Glucose and cholesterol levels, and also pulse rate, must be constantly maintained to limit the risk of complications.[8-10] Elevated insulin sensitivity, insulin resistance, and pancreatic cell failure are all associated with T2D, which can cause up to 50% tissue loss at diagnosis.[11] Teenagers lost -cells at a faster pace that might explain why those diagnosed at an early age have a greater likelihood of therapeutic failure.[12-15] SGLTi is a new type of diabetic medication and its analytical approach Glucose was reabsorbed from the glomeruli via the sodium-dependent glucose co-transporter inhibitors 1 and 2.[16-18] An SGLT2 inhibitor called empagliflozin has been shown to authorized for the purpose of those with type 2 diabetes in Europe, US, and Japan, among others places.[19] In individuals with T2D and CVD, empagliflozin showed cardio protective and Reno protective effects that were essentially independent of glycemic management.[20]

A modern technique called UPLC has given liquid chromatography a fresh start of life.[21] It may be used on particles with a diameter of less than 2mm to improve resolution, speed, and sensitivity.[22] In the meanwhile, Analytical labs are no exception to the trend that UPLC analysis enhances the quality of their products. High pressure (up to 100M Pa) is used in UPLC for separation and quantification.[23] Time and money are saved, solvent consumption is minimized, and more goods are evaluated using current resources.[21] Liquid-Liquid Extraction (LLE) is a process that was created and used to process the method. LLE is a traditional process that involves dispersing appropriate extraction and dispersant solvents into aqueous solutions, primarily from water and biological fluid samples. For HPLC-grade empagliflozin, the ideal solvents are ethyl acetate and water. Adjustments to the aqueous phase's pH and ionic strength have an impact on how well substances are extracted. The LLE extraction process with

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centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as nonpolar solvent and water is used as polar solvent for liquid-liquid extraction. LLE is a low cost extraction process compare to solid liquid extraction.[24]

MATERIALS AND METHODS

The raw materials of empagliflozin and empagliflozin D4 are procured from MTR lab, Chennai. Melting and solubility investigations are used to verify the samples. UPLC method that has given liquid chromatography a new lease of life. It may be used on particles with a diameter of less than 2 mm to improve resolution, speed, and sensitivity. The human plasma collected from human volunteers. The Ethyl acetate and water used in HPLC grade. This method helps to reduce the cost and time consumption. The structure of empagliflozin as shows the Figure 1.

Empagliflozin is used as Working Standard (WS) and it is soluble in Ethanol, DMSO, and dimethyl formamide. Appearance of empagliflozin is White powder and the colour is white to yellowish non-hygroscopic crystalline solid. Empagliflozin D4 is used as Internal Standard (IS) and it is soluble in methanol and DMSO. Appearance of empagliflozin D4 is White powder and the colour is white to yellowish non-hygroscopic crystalline solid.

Chromatography condition

The liquid chromatography contains Synergi 2.5µ Fusion-Reverse Phase 100A (100 mm×2.0 mm), 2.5 µm and maintained column temperature 50°C. The flow rate is 0.3 µL/min, and the injection volume is 10 µL. Auto sampler Temperature is 10°C. Empagliflozin parent and daughter ion condition set as 451.17 and 355.11. And for Empagliflozin D4 parent and daughter ion condition set as 455.15 and 358.21. Dwell (sec), Cone (Volts), Collision energy (ev) condition for WS and IS is 0.200, 20.00, 12.00.

Retention Time: Empagliflozin: 1.0 ± 0.35 duration
Empagliflozin D4: 1.0 ± 0.35 duration

Mass chromatography parameter

Waters Acquity UPLC-MS, model-Quattro Micro Mass, Software-Mass Lynx V 4.1 it contains ESI-Positive mode and the desolvation temperature is 500°C. The desolvation gas flow is 800L/hr, Capillary voltage is 4.00 kV and the cone gas flow: 50 L/hr.

Preparation of Reagents

Preparation of Buffer-1: [Water with 0.2% formic acid]

Transfer 500 mL of H_2O into a 1 liter of beaker. Make up the volume with water after adding 2 mL of formic acid. Mix thoroughly and sonicate in an ultrasonic bath for a short time. 0.2 m nylon membrane filter for filtration. Provide a batch number and document in the 'Solution Preparation Form'. Use this solution for four days from the date of preparation.

Preparation of Mobile Phase: [Methanol: Buffer: 1 (75:25, v/v)]

Add 250 mL of Buffer-1 and well blend after adding 750 mL of Methanol to a 1000 mL reagent bottle. Through a 0.2 m nylon membrane filter, filter. For a few minutes, sonicate in an ultrasonic bath. Fill out the "Solution Preparation Form" with a batch number and a document. Within four days of the preparation date, use this solution.

Preparation of Diluent – [H_2O: Methanol (50:50, v/v)]

Transfer 500 mL of methanol into a 1 L beaker that already has 500 mL of water in it. Add thoroughly and sonicate for a few minutes in an ultrasonic bath. Fill out the "Solution Preparation Form" with a batch number and a document. Use this solution for four days from the date of preparation.

Preparation of Analytical Solution (Empagliflozin stock solution) for CC

Accurately measure the weight of 2 mg of Empagliflozin into a 2 mL volumetric flask, and transfer. Include 1 mL of Methanol to dissolving and adding volume with same. Determine the final concentration of Empagliflozinin µg/mL as follows:

$$\frac{\text{Empagliflozin's weight (2 mg)}}{\text{2 mL}} \times \frac{\text{Strength (as basis)}}{100} \times \frac{\text{M1} \times 1000}{\text{M2}}$$

$$\frac{\text{Empagliflozin's weight (2mg)} \times \text{Strength (as Basis)} \times \text{M1} \times 1000}{\text{2ml} \times 100 \times \text{M2}}$$

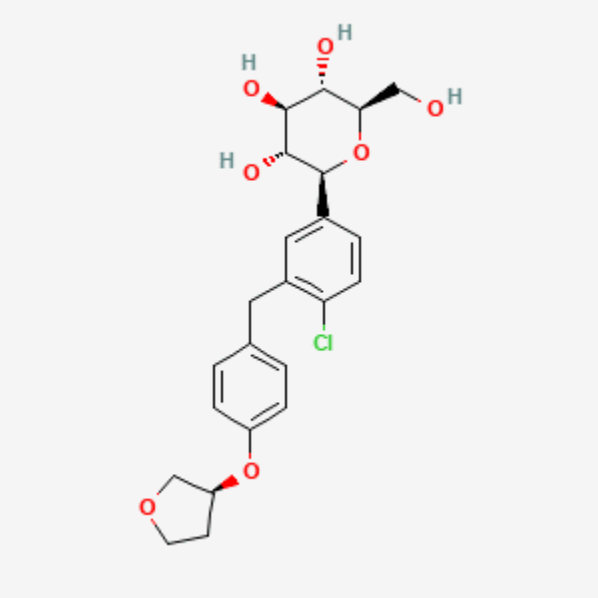

Figure 1: Chemical structure of Empagliflozin.

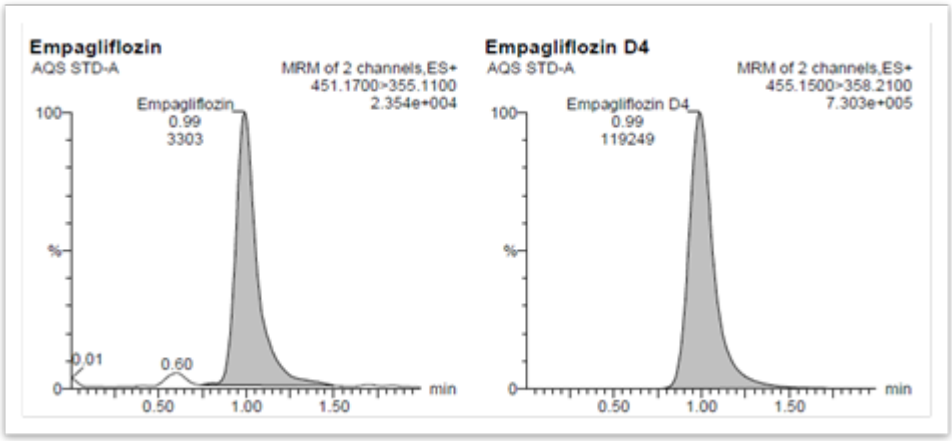

Figure 2: Representative chromatogram of standard A for empagliflozin.

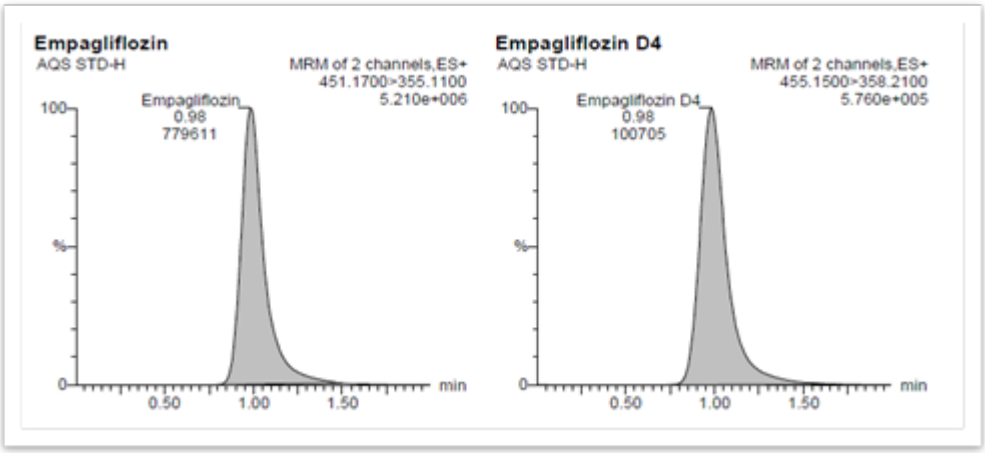

Figure 3: Representative chromatogram of standard H for empagliflozin.

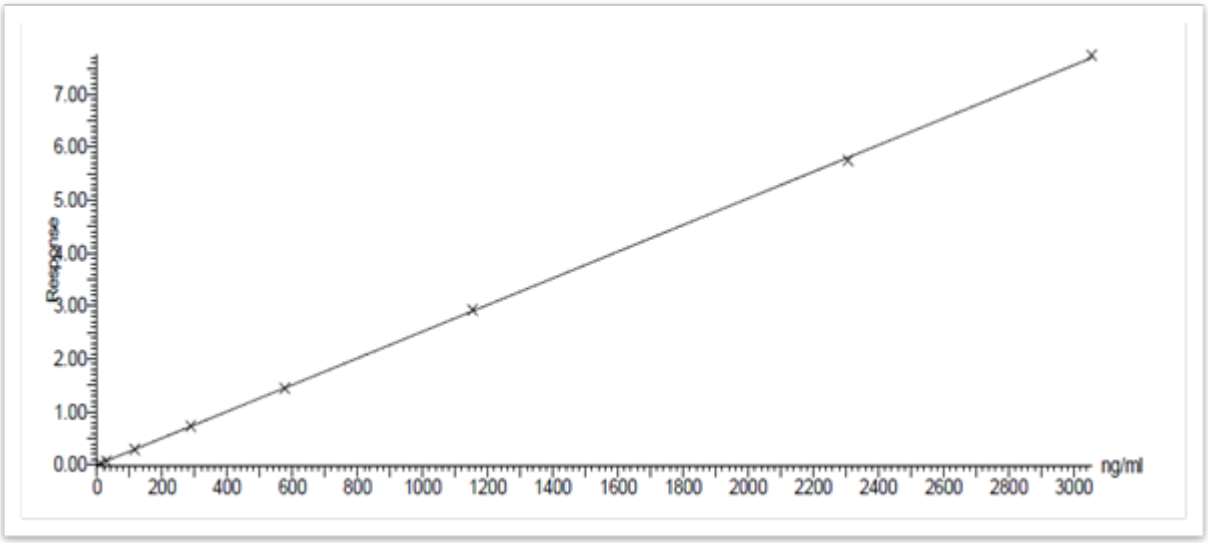

Figure 4: Calibration curve for empagliflozin.

Provide Equations in MS Equation

Where, M_1 & M_2 are the molecular weight.

Preparation of Empagliflozin D4 stock solution

Empagliflozin D4 should be accurately weighed at around 2 mg before being poured into a 2 mL volumetric flask. To dissolve, include 1 mL of Methanol (Table 3). Use it to fill the remaining space. Calculate the final Empagliflozin D4 concentration in g/ml as follows:

$$\frac{\text{Empagliflozin's weight D4 (mg)}}{\text{2 ml}} \times \frac{\text{Strength (as is basis)}}{100} \times \frac{M_1 \times 1000}{M_2}$$

$$\frac{\text{Empagliflozin's weight D4(mg)XStrength (as Basis)XM1X1000}}{\text{2ml X100XM2}}$$

Provide Equations in MS Equation

Internal standard Dilution

Develop the internal standard solution with diluent and empagliflozin D4 at a concentration of approximately 5 g/ml as instructed in (Table 1).

Spiked Calibration Curves for Standards

Add 0.200 mL of the stock aliquot of matching concentrations of the aforementioned stock dilutions of empagliflozin into a 10 mL volumetric flask. The remaining volume should subsequently be filled with pooled screened K2EDTA plasma. To obtain the concentrations indicated by the subsequent calibration curve in (Table 2).

Developing an empagliflozin stock solution for quality control

Empagliflozin WS should be accurately weighed at around 2 mg before being poured into a 2 mL volumetric flask. To dissolve, include 1 mL of methanol. Use it to fill the remaining space. Determine the empagliflozinin g/mL final concentration using the formula below:

$$\frac{\text{Empagliflozin's weight (mg)}}{\text{2ml}} \times \frac{\text{Strength (as is basis)}}{100} \times \frac{M_1 \times 1000}{M_2}$$

$$\frac{\text{Empagliflozin's weight (mg)XStrength (as Basis)XM1X1000}}{\text{2ml X100XM2}}$$

Provide equations in MS Equation

Where, M1 & M2 are the molecular weight of Empagliflozin (Free). Quality Control (QC) Samples.

RESULTS AND DISCUSSION

Empagliflozin was quantified for the duration of human plasma's concentration range of 10.2172 to 3075.213 ng/ml using a sensitive and specific UPLC-MS/MS technology that was devised and validated. Our research aims to create a validated method for UPLC MS/MS-based empagliflozin plasma measurement. The Internal Solution (IS) of empagliflozin D4. Liquid-liquid extraction was used to separate the samples on a Synergi 2.5 Fusion-RP 100A (100 mm2.0 mm), 2.5 m column. The ideal solvents for empagliflozin are methanol and water at HPLC grade. Performing LLE extraction with centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as nonpolar solvent and water is used as polar

Table 1: Preparation of Internal Standard solution for Empagliflozin D4.

Stock Concentration (µg/mL)	Stock Aliquot (mL)	Diluent (mL)	Final Volume (mL)	Final Concentration (µg/mL)
993.8445	0.101	19.899	20.000	5.0189

Table 2: Preparation of Empagliflozin spiked calibration curve standards:

Stock CCID	Stock Concentration (µg/mL)	Stock Aliquot (mL)	Plasma Added (mL)	Final Volume (mL)	Final Concentration (µg/mL)	Spiked SCCID
STD-A	10.188	0.200	9.800	10.000	10.2172	STD-A
STD-B	28.942	0.200	9.800	10.000	28.7211	STD-B
STD-C	115.306	0.200	9.800	10.000	114.7023	STD-C
STD-D	288.266	0.200	9.800	10.000	290.6332	STD-D
STD-E	576.532	0.200	9.800	10.000	574.5374	STD-E
STD-F	1153.062	0.200	9.800	10.000	1162.021	STD-F
STD-G	2306.124	0.200	9.800	10.000	2284.641	STD-G
STD-H	3054.468	0.200	9.800	10.000	3075.213	STD-H

Table 3: Preparation of stock dilution Empagliflozin of QC.

Stock Concentration (µg/mL)	Stock Aliquot (mL)	Diluents Added (mL)	Final Volume (mL)	Final Concentration (µg/mL)	Stock QCID
2296.855	0.225	1.775	2.000	2221.6796	AQ-HQC
1148.428	1.000	1.000	2.000	1143.1551	AQ-MQC
287.107	1.000	1.000	2.000	284.1430	AQ-INTQC
28.711	0.100	1.900	2.000	30.5438	AQ-LQC
10.214	0.705	1.295	2.000	12.4002	AQ- LOQQC

Table 4: K_2EDTA plasma screening for empagliflozin and internal standard.

Plasma Sample- ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio (≥5)
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS (<5%)	Analyte/IS	Analyte
Sample-I	8	11	1476	25563	0.542	0.043	0.0577	104.837
Sample-II	3	2	1370	24416	0.219	0.0082	0.0561	175.202
Sample-III	1	1	1497	25663	0.0668	0.0039	0.0584	113.093
Sample-IV	5	7	1466	23675	0.3411	0.0296	0.0619	207.594
Sample-V	0	1	1247	21974	0	0.0046	0.0568	33.822
Sample-VI	0	1	1397	23216	0	0.0043	0.0602	24.604
Sample-VII(H)	0	1	1359	23885	0	0.0042	0.0569	108.656
Sample-VIII(L)	0	0	1284	23865	0	0	0.0538	97.911
Sample-IX(Hep)	1	1	1460	23900	0.0685	0.0042	0.0611	60.266
				Mean	0.13748	0.01132	0.0581	
						SD	0.002591	
% of Lots passing =	100	%				%CV	4.46	
						Result	Pass	

Table 5: Carry over test for empagliflozin and empagliflozin D4.

	Sample ID	Analyte peak area	IS peak area
	Extracted blank	6	2
	Extracted LLOQ+IS	420	14862
	Extracted ULOQ+IS	132634	12250
	Extracted blank-I	3	1
	Extracted blank-II	5	18
	% Carry Over from Blank I	-0.71	-0.01
	% Carry Over from Blank II	-0.24	0.12
	Result for blank - I	Pass	Pass
	Result for blank - II	Pass	Pass

Table 6: Precision and Accuracy study for empagliflozin.

QC ID	LOQQC	LQC	INTQC	MQC	HQC
Actual Concentration (µg/mL)	10.214	28.711	287.107	1148.428	2296.855
Calculated Concentrations (µg/mL)	12.4002	30.5438	284.143	1143.1551	2221.6796
	10.6959	30.2529	286.153	1155.8118	2217.1241
	10.3007	29.3731	286.532	1116.3726	2183.178
	10.4723	30.4534	284.3421	1132.3971	2229.6597
	11.2448	29.039	285.453	1164.0011	2235.9214
	11.6174	30.4507	286.6755	1146.7779	2355.1806
Mean	11.12188	30.01882	285.54977	1143.08593	2240.45723
SD	0.796555	0.645411	1.099342	16.975751	59.121158
%CV	7.16	2.15	0.38	1.49	2.64
%Nominal	108.89	104.56	99.46	99.53	97.54

Table 7: Summary of Experimental Parameters and Results of Validated LC-MS/MS Method for the Quantification of empagliflozin in K_2EDTA human plasma.

Sl. No.	Experimental Parameters	Acceptable Range/Criteria (in %)	Result Obtained (in %)
1	Specificity and Selectivity	>80	100%
2	Recovery	≤110%	Analyte -85.3%, IS-87.05%
3	Carry Over Test	Analyte < 20 IS<5	Analyte - -0.71, IS- -0.01
4	Precision and Accuracy	80 ± 115%	LOQQC-109.17, LQC-103.72, INTQC-99.06, MQC-99.13, HQC-97.15

solvent for liquid-liquid extraction. LLE is a low cost extraction process compare to solid liquid extraction. The developed method is accurate, precise, sensitive method for determination of empagliflozin from human plasma solution. The reported technique is suitable for bioequivalence and pharmacokinetic studies.

Biological Matrix

The screening was conducted on the blank K2EDTA lots of human plasma. Micro Therapeutics Research Labs Pvt Limited in Chennai provided a plasma sample.

Standard calibration curve and quality assurance samples

Standards and quality control for curve calibration They were all produced in accordance with ICH recommendations and kept between -70° and 15° and -20° and 5°C.

Validation and Characteristics of Method

Specificity and selectivity

In order to test the specificity and selectivity, One lot of heparin plasma, one lot of lipemic plasma, and one lot of hemolyzed plasma were used, totaling six lots of blank plasma,confirming that the acceptance requirements were fulfilled and the sample passed the test (Table 4). Selectivity and Specificity. The matrix lots under study showed acceptable S/N intensities, with Signal-to-Noise ratios ranging from 24.604 to 207.594.

Carry Over Test

Carryover is determined as the proportion of peak area that is seen in a processed blank plasma that is injected in triplicate following a processing ULOQ calibration standard that was created from a PA batch sample. Internal standard and empagliflozin did not exhibit any discernible carryover (Table 5).

Linearity

To calculate the linearity concentration, an 8 point standardised calibration curve is prepared in human plasma. Using empagliflozin D4 as an internal standard and a CC curve, the concentration of empagliflozin varied from 10.2172 to 3075.213 µg/mL. using empagliflozin D4 as IS and for CC to the curve in (Figure 4).

Accuracy and precision

Six samples of the LLOQC, LOC, MQC, and HQC samples were examined to evaluate the assay's precision and accuracy (Table 6). Refer to Figure 2 for information regarding chromatogram A. Hare references in (Figure 3).

Empagliflozin recovery studies

The recovery of empagliflozin was used to evaluate the contrasting detector responses from un-extracted aqueous samples at three various levels with detector responses from low, moderate, and extremely high quality control samples of analyte extracted from extracted CC and QC samples from the PA batch. The average recovery rate for empagliflozin was 85.30 percent. The empagliflozin percent CV was 6.52 percent at 3 distinct QC levels. An average of 87.05 percent of the IS was recovered for empagliflozin D4.

CONCLUSION

A selective and sensitive UPLC MS/MS is recommended by ICH guidelines, technique for determining the amount of empagliflozin in human blood at concentrations between 10.2172 to 3075.213 ng/ml was developed. It was successfully validated using the experimental parameters, and Table 7 displays the results. The mobile phase and multiple liquid extractions are used in this single compound approach. In contrast to solid liquid extraction, LLE is a low-cost extraction method. For the detection of empagliflozin in a solution of human plasma, a sensitive, accurate, and exact approach has been established. Bioequivalence and pharmacokinetic investigations can be conducted using the reported approach.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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