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Validation of a novel UPLC-MS/MS method for estimation of metformin and empagliflozin simultaneously in human plasma using freezing lipid precipitation approach and its application to pharmacokinetic study

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Validation of a novel UPLC-MS/MS method for estimation of metformin and empagliflozin simultaneously in human plasma using freezing lipid precipitation approach and its application to pharmacokinetic study

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ABSTRACT

A fast, sensitive one step UPLC ESI-MS/MS method was successfully applied for the simultaneous estimation of two concurrently administrated antidiabetic drugs, Metformin (MET) and Empagliflozin (EMPA) in human plasma. Metformin-d6 (MET-d6) and Empagliflozin-d4 (EMPA-d4) were utilized as internal standards. Extraction of the analytes from the human plasma was performed through acetonitrile precipitation technique followed by freezing the precipitated plasma proteins and lipids to minimize the matrix effect. Chromatographic analysis was developed on Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 50 mm) using isocratic elution mode. A mobile phase of formic acid (0.01 %): acetonitrile (70:30 v/v) with a flow rate of 0.3 mL/min achieved optimum separation. Multiple reaction monitoring (MRM) in positive ion mode, with transitions at (*m/z*) 130.14 →71.08 for (MET), 451.72 →71.29 for (EMPA), 136.03 →77.02 for (MET-d6), and 455.43 → 75.05 for (EMPA-d4) was used for quantification. The obtained linearity covered the concentration ranges of 10–1500 ng/mL and 2.0–250.0 ng/mL for MET and EMPA, respectively. The run time of the proposed Method didn't exceed 3.0 min allowing faster analysis and determination of larger number of samples per day without affecting accuracy and sensitivity. The presented chromatographic method could be successfully applied in pharmacokinetics studies and therapeutic monitoring of MET and EMPA in patients' plasma administrating fixed dose combination of both drug with high reproducibility and ruggedness.

1. Introduction

Metformin hydrochloride (MET), a biguanide, exhibits antihyperglycaemic effects via increasing the activity of 5′Adenosine monophosphate activated protein kinase enzyme (AMPK). Activation of AMPK results in enhancing insulin sensitivity of body cells, increasing the glucose utilization and uptake peripherally, decreasing the glucose absorption from the gastrointestinal tract and reducing hepatic glucose production by inhibiting gluconeogenesis and glycogenolysis [1]. While Empagliflozin (EMPA) inhibits the sodium glucose co-transporter subtype-2 protein (SGLT2) in the kidney proximal tubule. Inhibition of SGLT2 suppresses the reabsorption of glucose and sodium and increases their urinary excretion through the kidneys, thus lowering plasma glucose level as well as blood pressure. [2] chemical structures of both drugs are shown in Fig. 1.

US Food and Drug Administration and European Medicines agency have approved the combination of MET and EMPA fixed-dose tablet in 2015, which provide two synergetic glucose-lowering mechanisms to enhance the glycemic control and better management of Type II non-insulin dependent diabetes mellitus as an adjunct to exercise and healthy life style [3,4].

One of the major challenges that may face the analyst, when conducting an LC–MS/MS method, is the influence of co-eluting components at the chromatographic retention time of the targeted

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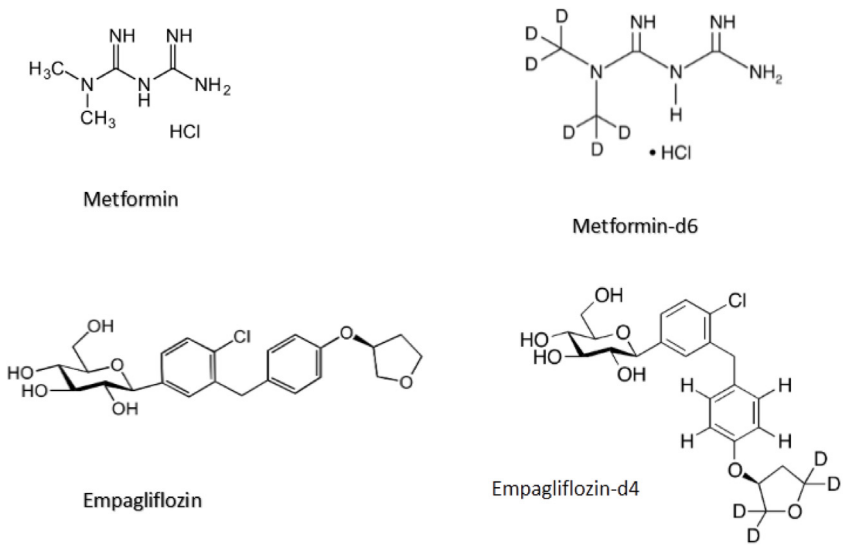

Fig. 1. Structural representation of MET, MET-d6, EMPA and EMPA-d4.

analytes; matrix effect, where it may significantly affect the analytes ionization including ion suppression or enhancement. For example, the co-elution of phospholipids, which are considered to be the main components in biological fluids and cell membranes, may contribute to suppression of the ionization and enhancement of the signals. Thus the elimination of phospholipids from the biological fluids and samples is highly recommended to improve sensitivity, reproducibility and reliability of the bioanalytical methods [5–8].

Different methods were suggested to get rid of the lipids through extraction, including freezing out or low temperature precipitation. The Method is based mainly on the low solubility of nearly 90 % of lipids as phospholipids and triglycerides in acetonitrile so it is used for extraction and the extract is then freezed at low temperature. Upon centrifugation, most of lipids and proteins are precipitated at the flask bottom in the form of condensed pale yellow lump [9–11].

The Literature survey revealed several methods for detection of MET either in single form [12,13] or in combination with other antidiabetic drugs as Alogliptin [14], Sitagliptin [15], Canagliflozin [16], and Dapagliflozin [17]. Similarly, different Methods were reported for detection of EMPA either alone [18] or in presence of other drugs as Linagliptin [19], Glimepiride [20], Pioglitazone [21] and Canagliflozin with Dapagliflozin [22].

Both drugs were estimated simultaneously in their pharmaceutical dosage forms using UPLC-MS/MS [23] and in human plasma using LC–MS/MS and solid phase extraction (SPE) to eliminate matrix effect. The SPE technique is expensive and lack selectivity [24], while methods involving protein precipitation followed by evaporation of supernatant was found to increase the matrix effect due to the concentration of the matrix components [25]. Other method used an adduct ion for EMPA quantitation [26] while to create a more robust and applicable LC/MS/MS method, it is preferable to avoid adduct formation [27].

The aim of the presented work is to develop and validate a simple, cost effective and fast UPLC-MS/MS bioanalytical method for simultaneous determination of MET and EMPA in human plasma with high accuracy and precision. The matrix effect was eliminated, during sample preparation, through protein precipitation technique using acetonitrile at low temperature. The method validation was performed in accordance to current guidelines of United States Food and Drugs Administration (FDA) and application to a pharmacokinetic study on healthy Egyptian volunteers was successfully achieved.

2. Experimental

2.1. Chemicals and reagents

Metformin hydrochloride and Empagliflozin hydrochloride with purity 99.74 % and 99.75 % was kindly obtained from Auro laboratories limited (Mumbai, India) and Century Pharmaceuticals Limited (Gujarat, India), respectively. Metformin hydrochloride D6 (98.10 %) and Empagliflozin hydrochloride D4 were purchased from Toronto Research Chemicals (Canada). Extra pure Formic acid (Scharlau), HPLC grade acetonitrile and methanol (Merk). De-Ionized ultrapure water was supplied via a Millipore Milli-Q® Integral 3 Water Purification System (USA). Blank plasma (kept at −80 °C) was kindly obtained from Vacsera (Giza, Egypt) and from volunteers participating in the clinical study.

2.2. Instrumentation and chromatographic conditions

Waters Acquity H-Class TQD-UPLC system (USA) conjugated with an electrospray ionization and operated in positive ionization mode. The chromatographic separation was performed on an Acquity UPLC BEH C_{18} column (50 × 2.1 mm, 1.7 μm) whose temperature was set at 30 °C. The mobile phase consisted of formic acid (0.01 %): acetonitrile (70: 30, v/v) at a flow rate of 0.3 mL/min using isocratic elusion mode and 10 μL injection volume. The source parameters were adjusted as following: capillary voltage, 2.0 kV; cone gas flow, 10 $L.hr^{-1}$; desolvation gas flow, 500 $L.hr^{-1}$; source temperature, 150 °C; desolvation temperature, 500 °C; The cone voltage 18 V for MET and MET-d6, 16 V for EMPA and EMPA-d4; The collision energy were 18 eV for MET and MET-d6, 24 eV EMPA and EMPA-d4. MRM mode of the transitions *m/z* 130.14–71.08 for MET, *m/z* 136.03–77.02 for MET-d6, *m/z* 451.72 to 71.29 for EMPA and *m/z* 455.43–75.05 for EMPA-d4. The data acquisition and instrumental control was performed via Mass Lynx software (version 4.1).

2.3. Preparation of standard stocks, calibrators, and quality control samples

Primary stock solutions of MET and EMPA were separately prepared in concentration of 1000.00 μg/mL using methanol as solvent. Secondary stock solutions for both drugs in concentration of 100.00 μg/mL were separately prepared by transferring and diluting suitable aliquots from the previously prepared primary

stock solutions using methanol. Eight different working standard solutions of MET and EMPA were prepared by accurately taking suitable volumes from its primary and secondary stock solutions into 2 different sets of 10-mL volumetric flasks to prepare working solutions of concentrations of 1.0, 2.0, 5.0,10.0, 40.0, 80.0, 120.0 and 150.0 μg/mL for MET, and 0.2, 0.4, 1.0,5.0, 10.0, 15.0, 20.0 and 25.0 μg/mL for EMPA, which were used for spiking the calibration standards. However, Solutions at concentrations 1.0,3.0, 45.0 and 130.0 μg/ mL for MET and 0.2,0.6, 7.5 and 22.5 μg/mL for EMPA were prepared using separate weightings for spiking QC samples. Stock solutions of the internal standards (IS), MET-d6: 75.0 μg/mL and EMPA-d4: 48.0 μg/mL, in methanol were separately prepared. The IS mixture solution (MET-d6: 0.050 μg/mL and EMPA-d4: 0.025 μg/mL) was prepared in acetonitrile used in sample preparation. Eight-point calibration standards and QC samples were prepared by spiking the blank human plasma with appropriate concentration of MET and EMPA.

Plasma calibration standards samples were prepared at concentrations of 10.0, 20.0,50.0, 100.0, 400.0, 800.0, 1200.0 and 1500.0 ng/mL for MET, and 2.0, 4.0, 10.0,50.0, 100.0, 150.0, 200.0 and 250.0 ng/mL for EMPA. Quality control samples were prepared at four concentrations level; lower limit of quantification (LLOQ) 10.0 ng/mL for MET and 2.0 ng/mL for EMPA, quality control low (QCL) 30.0 ng/mL for MET and 6.0 ng/mL for EMPA, quality control medium (QCM) with 450.0 ng/mL for MET and 75.0 ng/mL for EMPA, and quality control high (QCH) 1300.0 ng/mL for MET and 225.0 ng/mL for EMPA. The spiked Calibration standards and QC samples were stored at −80 °C.

2.4. Plasma sample preparation

0.3 mL of acetonitrile containing IS of MET-d6 (0.050 μg/mL) and EMPA-d4 (0.025 μg/mL) was added to 0.15 mL of plasma sample and vortexed for 30 s. The samples were freezed for 10 min at -80 C○. After thawing, the samples were centrifuged for 10 min at 5000 rpm. 0.20 mL of the obtained supernatant was accurately transferred to another tube containing 0.20 mL formic acid (0.01 %.) and vortexed for 30 s. 10 μL of the prepared sample was injected into the system UPLC TQD/MS.

2.5. Method validation

The Method validation was performed according to the US FDA guidelines 2018 with respect to linearity, selectivity, accuracy and precision, carry-over, dilution integrity, recovery, matrix effect and stability.

2.5.1. Selectivity

The Method selectivity was evaluated using six different sources of human blank plasma samples to assess any interference from the endogenous components of plasma. In addition, the impact of hemolyzed samples, lipemic samples and internal standards were investigated.

2.5.2. Linearity and range

Six different calibration curves were constructed over three days using eight concentration levels. The concentrations used for MET were 10.0, 20.0,50.0, 100.0, 400.0, 800.0, 1200.0 and 1500.0 ng/mL while the concentrations used for EMPA were 2.0, 4.0, 10.0,50.0, 100.0, 150.0, 200.0 and 250.0 ng/mL. A blank sample and zero sample with IS were tested with each calibration curve. To construct the calibration curves, the ratio of peak areas (Analyte/IS) were plotted against the nominal calibration standards concentrations using the weighted least squares linear regression Method ($1/x^2$).

2.5.3. Accuracy and precision

The intra- and inter-day precision were assessed via analyzing six QC samples at four different levels (LLOQ, QCL, QCM and QCH) at three independent runs over three days. The percent coefficient of variation (%CV) of the concentrations at each level should not more than 15 %, except at the LLOQ not more than 20 %. The Method accuracy was displayed as relative error which should not be more than ±15 %, except LLOQ was set at ±20 %.

2.5.4. Carry-Over

The Carry-over was investigated through determination of three blank plasma samples after the ULOQ to assure that precision and accuracy were not affected. It shouldn't exceed 20 % of the analyte peak response at the LLOQ and 5% of the IS peak response.

2.5.5. Dilution integrity

The dilution integrity was tested to assure that samples dilution had no influence on the accuracy of the measurements. Six replicates of QCs dilution were prepared separately at concentrations above the ULOQ and diluted two and four folds with blank samples. The diluted QCs accuracy and should be within ±15 % of the nominal concentration and the precision (%CV) should also be within 15 %.

2.5.6. Recovery

The extraction recovery of MET, EMPA and IS from human plasma was evaluated through a comparison between the analytes peak responses, extracted from QC samples, at different levels (QCL, QCM and QCH) versus the analytes response, from spiked post-extracted blank plasma samples, at equivalent concentration levels

2.5.7. Matrix effect

Matrix Effect was investigated using six replicates at a QCL and QCH including hemolyzed and lipemic ones. The matrix factor (MF) for MET, EMPA and IS was determined for each lot of matrix as illustrated in Eqs. 1 and 2. The IS normalized MF was also determined as illustrated in Eq.s 3. The % CV of the IS-normalized MF obtained from 6 matrix lots shouldn't exceed 15 %.

$$\mathrm{MF_{analyte}} = \frac{\text{Analyte peak area in post} - \text{extracted spiked sample}}{\text{Analyte peak area in mobile phase}} \quad (1)$$

$$\mathrm{MF_{IS}} = \frac{\text{IS peak area in post} - \text{extracted spiked sample}}{\text{IS peak area in mobile phase}} \quad (2)$$

$$\mathrm{IS_{-norm}\ MF} = \frac{\mathrm{MF_{analyte}}}{\mathrm{MF_{IS}}} \quad (3)$$

2.5.8. Stability

The stability tests were conducted with the human plasma containing MET and EMPA through the analysis of 3 replicates at high and low concentrations. The analytes stability in plasma was studied after 24 h at room temperature. Post-preparative QCs samples were kept at 4 °C in the auto-sampler and assessed after 72 h from the initial injection. The QC samples were assessed after three freeze thaw cycles, stored for more than 12 h at −80 °C and thawed at room temperature. The QC samples were frozen for 20 days at −80 °C. the stability of stock solution was assessed at high and low concentrations, stored at 4 °C for 24 h and 20 days. The results of stability QCs were compared against freshly prepared calibration curves and freshly prepared QCs. For each QC level, the mean concentration shouldn't exceed ±15 % of nominal concentrations.

2.5.9. Incurred samples reanalysis (ISR)

ISR is performed to ensure the reproducibility of the proposed bioanalytical method and verify the reliability of the measured actual sample concentrations. ISR was conducted via repeating analysis for a subject subset obtained from a given study on different days in separate runs. ISR was chosen randomly around the Cmax and some in the elimination phase. The percentage difference of the results between the original value and the repeat value is determined with the following equation:

%difference = ((Repeat–Original)/Mean)x100.

The percent difference should be not more than 20 % for at least 2/3 of the repeated samples.

2.6. Pharmacokinetic study

The proposed method was developed and applied for estimating samples obtained from six healthy Egyptian adult volunteers after administrating orally a single dose of Synjardy ™ tablet labelled to contain 12.5 mg EMPA and 500 mg MET (Boehringer Ingelheim, Canada). Prior to participation in the study, the laboratory tests and medical history of all the volunteers were screened for any disease or abnormal findings. Their ages ranged between 18–55 years, Body Mass Index ranged between 19–30 K g.m^2. The study was conducted in accordance to guidelines of the ICH principles for Good Clinical Practice and Helsinki Declaration for biomedical researches involving human subjects which recommend that all of the participating volunteers should be informed about the purpose, protocol and any possible risk of the study before giving a written consent to participate. According to Egyptian Health ministry, it is obligatory to approve the protocol study by the ethical committee. In addition, International Center for Bioavailability, Pharmaceutical and Clinical Research (Cairo, Egypt) approved and reviewed all experimental procedures. Eating and drinking was prohibited for at least 12 h before the dosing except for water. To prevent the occurrence of hypoglycemic episodes, 240 mL of glucose solution (20 %) was coadminstrated with the drug products followed by the administration of 60 mL of glucose solution every 15.0 min for 4 h. Blood samples were collected into labelled heparin vacutainer tubes at pre-dose; 0.00, 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.33,3.67, 4, 5, 6,8, 10, 12, 16,24, 36, and 48 post dose. The collected samples were centrifuged immediately at 4000 rpm for 5.0 min. The plasma samples transferred into the labelled polypropylene tubes then stored at −80 °C until analysis. The study design was open label, single dose, randomized, one period and one treatment. The study pharmacokinetic parameters were C_{max}, T_{max}, $t_{1/2}$, Elimination rate constant, AUC_{0-t} and AUC_{0-inf}. Phoenix WinNonlin software 6.4, Pharsight®, USA was used. The informed consent has been taken from all participants in this study and they have agreed to use their clinical specimens in the planned research work

3. Results and discussion

3.1. Method development

It was a challenge to design a proper bioanalytical Method for simultaneous quantitation of analytes possessing different physical and chemical properties where different approaches in chromatographic separation and plasma extraction might be required.

3.1.1. Mass spectrometry optimization

For MET and EMPA, the mass parameters were tuned in both the positive and negative ionization mode. Due to the basic characters of MET, it could only be ionized in the positive mode. While for EMPA with its C-glycoside structure, the results revealed that positive ionization mode exhibited stronger signals and higher intensity than the negative ionization mode [28], Fig. 2. 0.3 μg/mL of the analytes standard solutions was infused into various mobile phases containing ammonium acetate, ammonium formate, MET showed $[M+H]^+$ ion at *m/z* 130.00 while EMPA showed $[M+H]^+$ ion at *m/z* 451.00 and ammonium adduct ion $[M+NH_4]^+$ ion at *m/z* 468.00 as previously reported [29,30] Fig. 2. Hence, ESI^+ mode was suitable for detecting MET and EMPA and the IS. The mass spectrometric parameters were optimized including the energy of collision and product ions were chosen while infusing the standard solutions in 0.01 % formic acid as following; 71.08, 77.02, 71.29, 75.05 for MET, MET-d6, EMPA and EMPA-d4, respectively where reproducible responses were obtained.

3.1.2. Chromatographic conditions optimization

MET is small in size, polar in nature and hydrophilic, thus poorly retained on the reversed phase columns. In contrast, EMPA could be more retained because of its lower polarity. To achieve sharp peaks, strong signals, and accepted resolution, different analytical columns were tried including; Acquity BEH C_{18} column (1.7 μm, 2.1 × 50 mm), Acquity HSS C_{18} column (1.8 μm, 2.1 x 100 mm), Agilent Zorbax SB-C_{18} column (5.0 μm, 4.6 x 50 mm). In addition, different organic solvents with variable ratios including; methanol and/or acetonitrile with aqueous buffer (water and 0.1 % formic acid, 0.01 % formic acid or 0.1 % Ammonia) were tested as mobile phase. For assays with high robustness, mobile phases containing ammonium acetate, ammonium formate were not used to avoid the formation of competitive adduct ions. Mobile phases containing water or ammonia buffer gave good peak response for EMPA and low or no peak response for MET. The chromatographic analysis was best achieved on an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 50 mm) using formic acid (0.01 %) and acetonitrile (70:30, v/v) as mobile phase and flow rate of 0.3 mL/min.

3.1.3. Sample preparation optimization

It was impossible to extract MET and EMPA simultaneously from human plasma through liquid-liquid extraction techniques owing to its hydrophilic nature (Log P= -0.918) and highly water solubility hindering its migration to the organic layer. The Direct protein precipitation technique with acetonitrile was found to be the most convenient one for extraction of the both analytes simultaneously. MET estimation in plasma was very tedious due to its poor retention on reversed-phase columns and probability of plasma matrix components interfering with its evolution. 77%–96% of the lipids matrix as phospholipids and triglycerides could be approximately eliminated via acetonitrile precipitation induced by freezing. In this study, the protein content in 0.150 mL of human plasma sample was precipitated by 0.3 mL of acetonitrile containing IS and vortexed for 30 s followed by 10 min freezing at -80 C○. After thawing, the samples were centrifuged for 10 min at 5000 rpm to induce the formation of a dense precipitate of lipids and proteins. The clear supernatant was diluted with formic acid (0.01 %) in ratio 1:1 to improve the peak shape. Minimum matrix effects and maximum extraction recovery were achieved. One of the goals of this research is to apply the freezing lipid precipitation approach in human plasma to eliminate plasma phospholipids which causing the matrix effect and use this approach for extraction of polar compound e.g. Metformin and non-polar compound e.g. Empagliflozin. Adding acetonitrile to human plasma sample precipitates plasma protein, then freezing for 10 min at -80 C○ or for 30 min at -20 C○ followed by centrifugation precipitates the plasma lipids components Fig. 3.

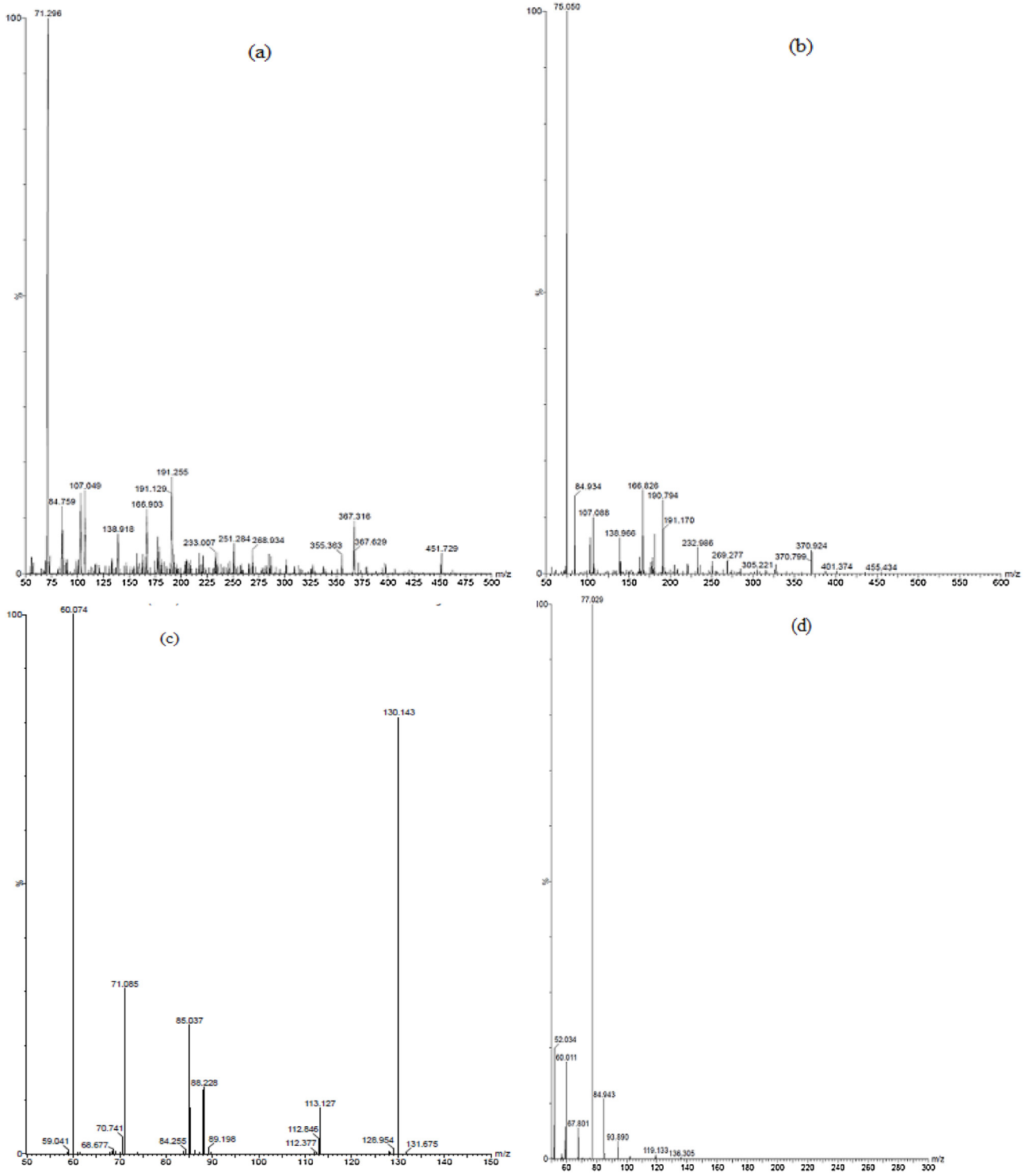

Fig. 2. The ESI-MS daughter ion scan of EMPA (a), EMPA-d4 (b), MET (c) and MET-d6 (d) in positive ionization mode.

3.2. Method validation

3.2.1. Selectivity

The chromatograms displayed in Fig. 4 indicate the absence of human plasma interference peaks at the analytes and IS retention times. The retention times of MET, MET-d6 (IS), EMPA and EMPA-d4 (IS) were 0.48, 0.47, 2.23 and 2.17 min, respectively.

3.2.2. Linearity and range of quantitation

The calibration graphs exhibited linearity over the concentration ranges of 10.0–1500.0 and 2.0–250.0 ng/mL for MET and EMPA, respectively. LLOQ for MET and EMPA were found to be 10.0 ng/mL for and 2.0 ng/ mL, respectively with the %RSD < 20 %.

The regression equation could be represented as following; Y = aX + b. in which Y is the peak area ratio of analyte/internal stan-

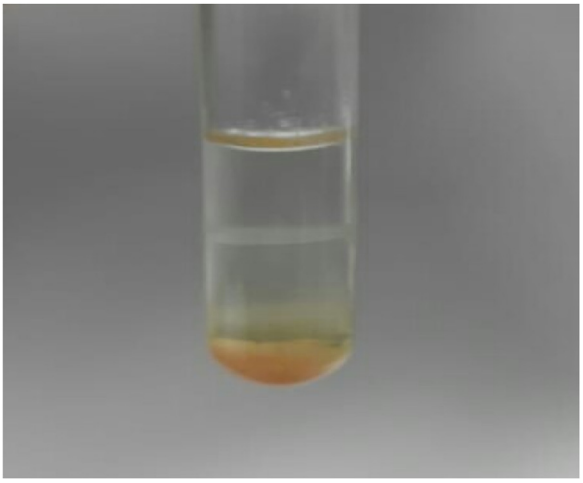

Fig. 3. Show the effect of freezing lipid precipitation approach on precipitation of 1.0 mL human plasma by 1.0 mL Acetonitrile, vortex30 sec. and freezing 10 min at -80 C° followed by Centrifugation 5.0 min at 4200 × g (Layers from bottom to top precipitate of plasma protein, precipitate of plasma lipid and bile pigments, slightly turbid layer with plasma lipid and very clear layer used for analytes determination).

dard while X is the corresponding concentration in ng/mL of the analyte under investigation. The mean equation of the calibration plots (n = 6) was represented as following;

$$\text{ForMTFY} = 0.0038X + 0.0111 r^2 = 0.9999 \text{SDofslope} = 0.00011 \text{SDofIntercept} = 0.00240.$$

$$\text{ForEPGY} = 0.0119X + 0.0093 r^2 = 0.9997 \text{SDofslope} = 0.00054 \text{SDofIntercept} = 0.00273.$$

3.2.3. Accuracy and precision

The intraday precision (% CV) ranged from 0.9 % to 5.63 % for MET and 2.11%–3.89 % for EMPA. However, the intraday accuracy (%) from the nominal concentration ranged from 90.62%–103.48% for MET and 90.38%–101.2% for EMPA. The interday precision (% CV) ranged from 2.74 % to 6.32 % for MET and 0.85%–6.75% for EMPA. Whereas, the interday accuracy (%) from the nominal concentration ranged from 93.42%–105.5% for MET and 95.21%–100.86% for EMPA as shown in Table 1.

3.2.4. Carry-Over

Interference peaks were absent at the analytes retention time in blank samples analyzed after injection of the calibration standards at ULOQ.

3.2.5. Integrity of dilution

The results of precisions (CV %) of diluted samples diluted lied between 0.62–2.53%, while the accuracy (%) results from the nominal concentration were within 93.93–98.67 % for the analytes.

3.2.6. Recovery

The mean recovery for MET and EMPA were calculated at QCL, QCM and QCH levels. It ranged from 90.22–92.58 %, and 92.81–97.89 %, for MET and EMPA, respectively. The mean recovery for internal standards at the three QC levels were ranged from 94.05–96.51%, and 95.40–97.14 %, for MET-d5 and EMPA-d4, respectively and results were presented in Table 2.

3.2.7. Matrix effect

The average factor of IS normalized matrix exhibited a range between 0.99 to 0.93 for MET relative to MET-d6 while it ranged between 1.04 to 0.98 for EMPA relative to EMPA-d4 at QCL and QCH, respectively. The obtained results indicated no significant matrix effect over the analytes ionization. The CV% of the IS-normalized MF ranged from 1.10 to 2.05 and 3.89–9.79 for MET and EMPA, respectively, indicating lack of a relative matrix effect on ionization.

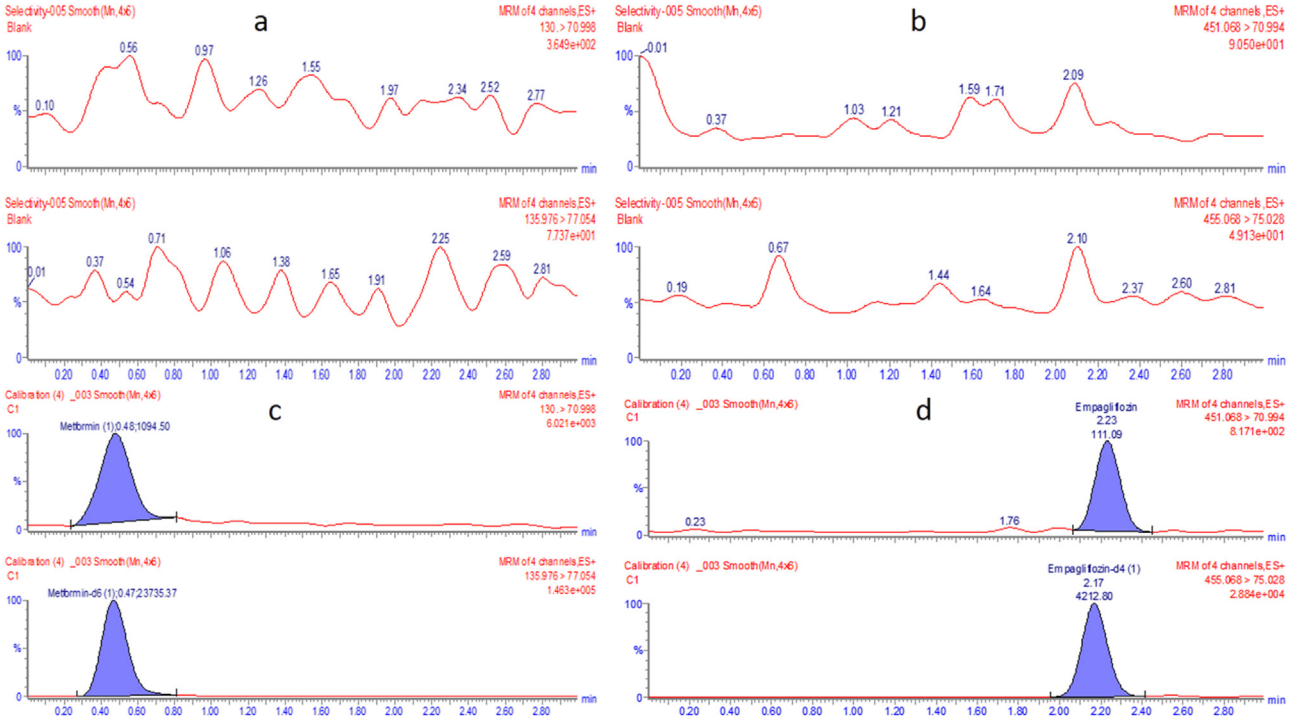

Fig. 4. MRM chromatograms of MET, MET-d6, EMPA and EMPA-d4 at blank samples (a, b), Spiked plasma at lower limit of quantitation (c) for MET(d) for EMPA.

Table 1
Accuracy and precision results for MET and EMPA.

Analyte	Level	nominal conc.	Intra-day (n = 6)		Intre-day (n = 18)	
			Precision (CV %)	Accuracy (%)	Precision (CV %)	Accuracy (%)
MET	LLOQ	10	5.63	90.62	6.32	97.33
	QCL	30	2.70	103.48	2.74	105.50
	QCM	450	0.90	95.46	4.93	93.42
	QCH	1300	1.42	97.68	3.21	97.47
EMPA	LLOQ	2	2.82	101.20	0.85	100.42
	QCL	6	3.89	90.38	4.52	95.21
	QCM	75	3.81	98.69	6.75	99.45
	QCH	225	2.11	97.78	6.71	100.86

Table 2
The recovery % in human plasma for Metformin and Empagliflozin in human plasma (n = 3).

	MTF				EPG			
	QCL	QCM	QCH	MTF-d6	QCL	QCM	QCH	EPG-d4
	88.82	92.93	93.38	97.39	91.88	94.50	99.3	98.42
	91.25	88.78	90.95	93.52	92.28	94.04	95.96	96.67
	94.85	89.04	90.26	93.04	94.01	90.05	97.81	96.31
Mean%	91.64	90.25	91.53	94.65	92.72	92.86	97.69	97.13
SD	3.03	2.32	1.64	2.39	1.13	2.45	1.67	1.13
CV%	3.31	2.57	1.79	2.52	1.22	2.64	1.71	1.16

Table 3
stability tests of MET and EMPA stability tests at different conditions.

Stability parameters	QC Level	MET		EMPA	
		Precision (CV %)	Accuracy %	Precision (CV %)	Accuracy %
Short-term stability at room temperature (24 h)	QCL	2.19	108.87	4.23	98.65
	QCH	0.89	96.76	2.13	91.67
Postoperative stability at 4 °C(72 h)	QCL	2.66	109.23	2.02	100.20
	QCH	0.42	100.16	1.64	101.19
Freeze–thaw stability at −80 °C (three cycles)	QCL	2.54	106.21	3.46	103.98
	QCH	1.25	99.56	2.35	99.64
Long term stability at −80 °C(20 days)	QCL	2.80	104.64	0.28	99.71
	QCH	0.94	99.02	0.88	95.97
Stock solution stability at 4 °C (20 days)	QCL	0.57	102.47	3.22	103.10
	QCH	0.63	102.37	1.14	99.89

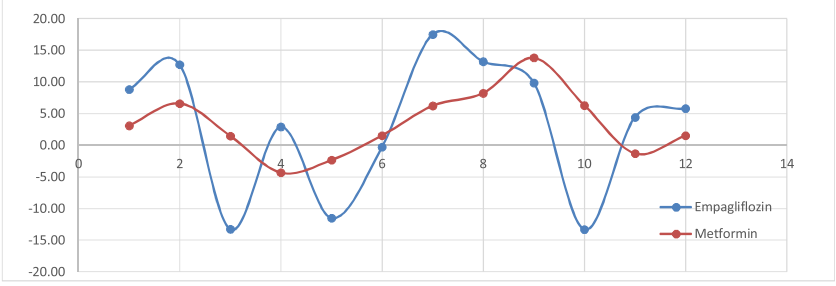

Fig. 5. Graphical representation of the results for 12 incurred samples reanalysis of Empagliflozin and Metformin.

3.2.8. Stability

Acceptable results for the stability testes were obtained as the change in concentrations was less than 15 % of the nominal concentrations which confirmed the stability of the processed samples. The post-preparative, freeze-thaw, short-term, long-term and stock solutions stability were investigated and the obtained results were summarized in Table 3.

3.2.9. Incurred samples reanalysis (ISR)

The ISR experiment was done to evaluate reliability of the study data. The percentage difference of the results between the original value and the repeated value was ranged from -13.32 to 17.51 % for the analytes. The results were presented in Fig. 5.

Table 4
Pharmacokinetic parameters of MET and EMPA after oral administration of single dose FDC of Synjardy ™ tablet to 6 Egyptian volunteers.

Parameters	Metformin (mean ± SD)	Empagliflozin (mean ± SD)
Cmax (ng/mL)	799.09 ± 222.61	166.77 ± 35.71
Tmax (hours)	3.0 ± 1.5	2.5 ± 1.5
T1/2 (hours)	6.18 ± 4.63	7.82 ± 2.20
AUC0-t (ng h/mL)	6704.73 ± 2130.37	1404.65 ± 237.51
AUC0-∞ (ng h/mL)	6831.91 ± 2176.12	1447.40 ± 250.06
Kel (h−1)	0.15 ± 0.06	0.09 ± 0.02

3.3. Application of method to pharmacokinetic study

The proposed bioanalytical UPLC-MS/MS method was successfully developed for simultaneous estimation of MET and EMPA

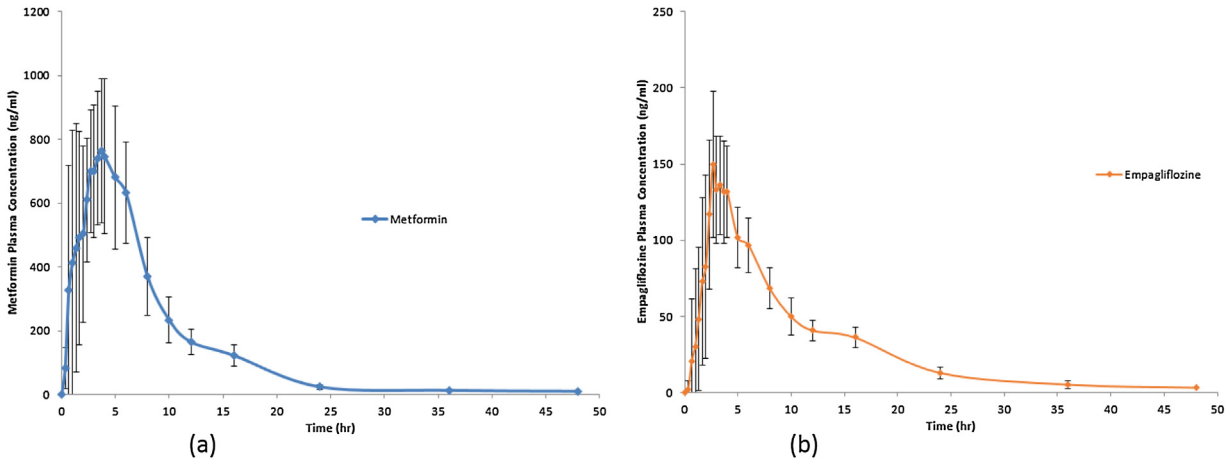

Fig. 6. Mean plasma concentration time profile of MET (a) and EMPA (b) after oral administration of single dose of FDC of Synjardy ™ tablet in human plasma.

Table 5
Comparative assessment of recently reported methods for Metformin and Empagliflozin determination in the human plasma.

	Shah et al. (2019) Ref. [24]	Wattamwar et al. (2020) Ref. [26]	Present work
Dose	5 mg Empagliflozin/500 mg metformin 150 mg Canagliflozin/1000 mg metformin 5 mg Dapagliflozin/500 mg metformin	12.5 mg Empagliflozin/1000 mg Metformin	12.5 mg Empagliflozin/500 mg Metformin
Extraction method	Ion-pair SPE using sodium lauryl sulphate. The eluates were evaporated till dryness followed by reconstitution by 0.2 mL of the mobile phase	SPE	Protein precipitation by Acetonitrile, then freezing followed by centrifugation to precipitate the plasma lipids components (for elimination matrix effect). Dilution 1:1
m/z	130.1/60.1 for Metformin 451.3/71.1 for Empagliflozin 462.1/267.1 for Canagliflozin 467.1/329.1 for Dapagliflozin With their istop labled internal standards	130.072/71.200 for Metformin 468.070/355.100 for Empagliflozin With their istop labled internal standards	130.14/71.08for Metformin 451.72/71.29 for Empagliflozin With their istop labled internal standards
Sample volume (mL)	0.3	0.1	0.15
LLOq (ng/mL)	metformin = 2.0 Empagliflozin = 1.5 Canagliflozin = 3.0 Dapagliflozin = 0.2	metformin = 10.09 Empagliflozin = 25.44	metformin = 10.00 Empagliflozin = 2.0
Method mean recovery	Metformin form 86.4%–89.9% Empagliflozin form77.4%–80.8%	Metformin form 74.06%–84.46% Empagliflozin form 81.61%–86.65%	Metformin form 90.26%–91.64% Empagliflozin form 92.72%–97.69%
Cmax(ng/mL)	Metformin = 678 Empagliflozin = 168	Metformin = 1764.14 ± 294.38 Empagliflozin = 179.36 ± 37.79	Metformin = 799.09 ± 222.61 Empagliflozin = 166.77 ± 35.71
Tmax (h)	Metformin = 3.5 Empagliflozin = 1.6	Metformin = 1.64 ± 1.51 Empagliflozin = 3.04 ± 1.42	Metformin = 2.67 ± 1.13 Empagliflozin = 2.37 ± 0.88
$T_{1/2}$ (h)	Metformin = 10.5 Empagliflozin = 13.4	Metformin = 4.43 ± 0.99 Empagliflozin = 6.58 ± 0.74	Metformin = 6.18 ± 4.63 Empagliflozin = 7.82 ± 2.20
AUC0-t (ng/mL h.)	Metformin = 5135 Empagliflozin = 1105	Metformin = 12968.66 ± 2033.63 Empagliflozin = 1691.44 ± 105.76	Metformin = 6704.73 ± 2130.37 Empagliflozin = 1404.65 ± 237.51
AUC0-∞ (ng/mL h.)	Metformin = 5442 Empagliflozin = 1235	Metformin = 13278.85 ± 2048.63 Empagliflozin = 1849.61 ± 139.18	Metformin = 6831.91 ± 2176.12 Empagliflozin = 1447.40 ± 250.06
Kel(h^{-1})	-	Metformin = 0.09362 ± 0.04 Empagliflozin = 0.10670 ± 0.01	Metformin = 0.15 ± 0.06 Empagliflozin = 0.09 ± 0.02
Vd/F (mL)	-	-	Metformin = 606.03 ± 221.74 Empagliflozin = 97.09 ± 18.18
Cl/F(L/h)	-	-	Metformin = 78.2 ± 19.76 Empagliflozin = 8.85 ± 1.15

in human plasma and validated for the pharmacokinetic study in six Egyptian healthy volunteers. The FDC tablets Syncarpy ™ were orally administrated under fasting condition. Fig. 6 illustrated MET and EMPA mean plasma concentration time profile. The mean pharmacokinetic parameters of MET and EMPA were presented in Table 4. The results were consistent with those obtained from previously published studies as summarized in Table5.

4. Conclusion

The proposed UPLC–MS/MS method presented a simple, selective, accurate rapid and cost effective method for simultaneous estimation of MET and EMPA in human plasma. Acetonitrile precipitation followed by freezing proved to be an efficient method for removing plasma proteins and lipids where the obtained results showed reproducibility and high analyte recovery. The data were validated in accordance to US FDA guidelines and could be applied for pharmacokinetic studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author statements

This paper has been achieved a new method and highly selective, sensitive for the simultaneous determination of Metformin and Empagliflozin in human plasma using freezing lipid precipitation approach based on novel UPLC-MS/MS. The presented chromatographic Method could be successfully applied in pharmacokinetics studies and therapeutic monitoring of MTF and EPG in patients' plasma administrating fixed dose combination of both drug with high reproducibility and ruggedness.

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