RESEARCH PAPER

Validation and Stability Assessments of Novel LC-MS/MS Method for Quantitative Determination of Bexagliflozin in Dog Plasma

Patel Smit Jayantilal[1,3]*, Mehta Hiralben[2], Shaik Basheer Ahmmad[3], Khan Nadeem[3]

[1]*Faculty of Quality Assurance, Parul University, Waghodia, Vadodara, Gujarat, 391760, India*
[2]*Department of Quality Assurance, Parul Institute of Pharmacy and Research, Parul University, Waghodia, Vadodara, Gujarat, 391760, India*
[3]*Department of Chemistry, Jai Research Foundation, Valsad, Gujarat 396105, India*
Author for correspondence: patelsmit87@gmail.com

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ABSTRACT

Background: Bexagliflozin is a novel sodium-glucose transporter 2 inhibitor approved by FDA for treatment and management of type-2 diabetes mellitus. The suggested LC/MS-MS method is appropriate for quantification of bexagliflozin in dog plasma.

Purpose: To establish and validate LC-MS/MS method for the quantification of bexagliflozin in canine plasma as dog is a non-rodent animal model for preclinical research.

Materials and Methods: The protein precipitation method was employed for extraction of analyte from dog plasma. Chromatographic separation was accomplished utilizing Thermo BDS Hypersil column (150 mm x 4.6 mm, 5 µm) with a mobile phase consisting of methanol: acetonitrile (50:50) and 10 mM ammonium formate in milli-Q water in a ratio of 90:10 (v/v) for isocratic elution. The runtime was 3.5 mins at flow rate of 0.7 mL/min and the injection volume was fixed at 10 µL. Rolipram was used as internal standard.

Results: The validated method showed significant sensitivity and demonstrated linearity in the range of 10 – 1280 ng/mL. The accuracy of the method was found to be 94–108%, while precision was found to be less than 9%. Lower limit of quantification was determined to be 10.38 ng/mL and bexagliflozin showed recovery of about 101%.

Conclusion: A simple, precise and reproducible LC-MS/MS bioanalytical method using protein precipitation extraction was developed for estimation of bexagliflozin in dog plasma. This developed method would be further utilized to support pharmacokinetics studies of analyte in biological matrix

Keywords: *Bexagliflozin, LC-MS/MS, Protein Precipitation, Dog Plasma*

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INTRODUCTION

Across the world, millions of people worldwide suffer with diabetes mellitus, a common and complicated chronic illness marked by elevated blood glucose level and projected that number of adults living with diabetes to rise 152 million by 2045, which is an increase of 68%[1]. T2DM is an increasing prevalent condition affecting population on both developed and developing countries[2]. In recent years, approaches targeting the renal SGLT2 have rapidly progressed as treatment options for T2DM. SGLT2 is low affinity and high capacity glucose transporter and exclusively present in proximal tubule (S1 and S2) where it facilitates co-transport of sodium and glucose[3]. SGLT 2 receptors account for 60-90% of glucose reabsorption during renal reuptake. Pre-clinical and clinical data suggest SGLT 2 inhibitors are promising glucose lowering agents but still challenge is exist to find the metabolically stable compound which can account for once a day treatment. The C-aryl glycosidase containing compound have been recognised as the most stable class of SGLT-2 inhibitors which subsequently given rise to discovery of remogliflozin, dapagliflozin, canagliflozin, bexagliflozin and sotagliflozin [4,5]. Among all these, in 2023 FDA has approved bexagliflozin for managing T2DM. Bexagliflozin is (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-(2-cyclopropyloxyethoxy)phenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol)) (Figure 1) that operate by supressing renal glucose uptake which consequently increases the amount of glucose eliminated through the urine[6].

**Author for Correspondence: patelsmit87@gmail.com*

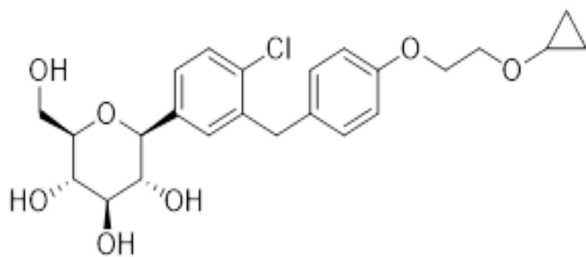

Figure 1. Structure of bexagliflozin

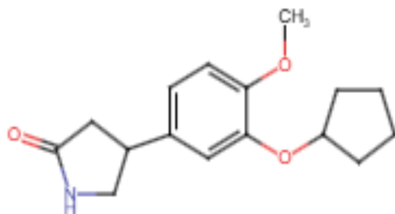

Figure 2. Structure of rolipram

Quantitative evaluation of drugs and their metabolite in different biological matrices should be robust during pre-clinical and clinical development. The literature survey revealed several RP-HPLC analytical techniques for quantification of bexagliflozin in a bulk form and pharmaceutical formulation[7-9]. As of now, there appears to be scarce of published LC-MS/MS based bioanalytical methods specifically for quantification of bexagliflozin, so current method was design and validated for quantifying bexagliflozin in canine plasma, employing rolipram as the internal standard. The structure of rolipram is given (Figure 2). This validation was performed as per recently revised ICH guidelines (ICH M10)[10].

MATERIALS AND METHODS

Chemicals and Reagents

Bexagliflozin (Batch Number: TC01-015A1) and rolipram (Batch Number: CS-RP-234) with purity of 99.75% and 98.82% were obtained from Transfo Chem (Maharashtra, India) and Clearsynth (Maharashtra, India), respectively. Methanol, acetonitrile, and ammonium formate were brought from Merck and deionized ultrapure water was supplied through Milli-Q system. Dog plasma (species: *Canis Familiaris*, strain: Beagle Dog (Male), Anticoagulant: K2EDTA, Batch number: PBS04112023) was procured from the Palamur Biosciences (Telangana, India). Six different lots were received and stored at frozen condition (-70 °C) till use.

LC-MS/MS Instrumentation

API 6500+ Mass Spectrometer coupled with Exion LC HPLC system was employed for analysis (positive ionization mode). Separation performed on Thermo BDS Hypersil [150 x 4.6 mm, 5 µm particle size] column at 40 ºC. Methanol: acetonitrile (50:50) and 10 mM ammonium formate in milli-Q water was used as mobile phase in a ratio of 90:10 (v/v) at a flowrate of 0.7 mL/min and 10 µL injection volume. The total run time was 3.5 minutes for each injection. Quantification was performed by using MRM mode with transitions of m/z 482.1→167.1 and 482.1→411.1 for bexagliflozin and m/z 276.16→ 131.0 for rolipram. The ion source parameters set for ESI positive mode; Collision energy and collision exit potential were maintained at 33 eV and 9 V (m/z 482.1→167.1), 18 eV and 16 V (482.1→411.1) for bexagliflozin and 30 eV and 12 V for IS curtain gas (CUR) was set at 40 psi, collision gas (CAD) at 10 psi, declustering potential (DP) 40 V, entrance potential 10 V, ion spray voltage (ISV) maintained 5500 V, nebulizer gas (GS1) at 50 psi, turbo gas (GS2) at 60 psi and 500 ºC temperature. The analyst software was utilized for data acquisition and instrument control (version number 1.7.1).

Standard Solution Preparation

Bexagliflozin and rolipram stock solutions were prepared in methanol. Working standards of bexagliflozin were established in diluent (50% methanol) and spiked into blank dog plasma to achieve concentration of 10 to 1280 ng/mL. Similarly, four QCs samples at 10.38, 30.62, 577.74 & 966.11 ng/mL were prepared in blank dog plasma and considered as LLOQC, LQC, MQC and HQC. Stock solution of rolipram (IS) was further diluted in diluent (50% methanol) to prepare working solution of (0.01 µg/mL).

Sample Extraction

Protein precipitation was employed for extraction. Concisely, 5 µL standard solution was spiked into 45 µL of plasma in a microcentrifuge tube, followed by 5 µL of IS addition (0.01 µg/mL). The mixture was vortex-mixed for 2 min, after which 1 mL of methanol was added to induce protein precipitation. The samples were then agitated at 2000 rpm for 10 min and subsequently centrifuged at 14,000 rpm at 4 °C for 10 min. The resulting clear supernatant was collected, and 5 µL was subjected to injection for analysis.

Method Validation

The current methodology validated as per ICH guidelines (ICH M10) by assessing system suitability, specificity and selectivity, determining linearity, evaluating precision and accuracy, stability, matrix effect, autosampler carryover and recovery[10-11].

System Suitability

System suitability test serves a critical role to ensure reliable and consistent performance of the bioanalytical method. Extracted plasma samples spiked with internal standard was prepared and injected six times from the same vial. Signal to noise ratio and % RSD of the peak area ratio were calculated.

Selectivity

Six separate drug free dog plasma samples were selected for investigation of interference of analytes at retention times. Selectivity of method was based on the chromatograms of blank plasma, spiked plasma with bexagliflozin and IS.

Specificity
Blank plasma fortified with analyte at ULOQ and IS separately to investigate specificity of the method. The interference at analyte retention time was monitored in the presence of sample of internal standard and vice versa.

Autosampler Carryover Test
Autosampler carryover was determined by processing and analyzing standard blank, LLOQ, and ULOQ standard in sequence followed by reinjection of the initial blank sample to check carryover. The second injection in series (LLOQ) was injected to find if any interference at retention time of bexagliflozin in standard blank.

Linearity
Eight calibration standards, including ULOQ and LLOQ were utilised to prepare calibration standard curves over the range of 10 – 1280 ng/mL. Peak area ratio of the bexagliflozin relative to IS against bexagliflozin nominal concentrations was plotted to construct regression equation with weighting factor ($1/x^2$).

LLOQ Determination
Method sensitivity was evaluated by LLOQ determination. LLOQ samples were processed by spiking bexagliflozin into six individual lots of dog plasma at LLOQ level with a working internal standard solution. These samples were analyzed with precision & accuracy samples and under a calibration curve along with batch quality control (QC) samples. Three batches of LLOQ determination were evaluated.

Precision and Accuracy
Six replicates of QCs (LLOQC, LQC, MQC, and HQC) representing the entire calibration curve range with concentrations at LLOQC (slightly higher than LLOQ concentration), LQC (approximately 3 times the LLOQ concentration), MQC (approximately 40-60% of ULOQ) and HQC (approximately 75-85% of ULOQ) were used for determination of precision and accuracy. To assess the ruggedness and potential transferability of the developed LC-MS/MS method, additional evaluations were carried out under deliberately varied conditions. The method was tested using two different analysts and columns on different days.

Reinjection Reproducibility
To verify the validity of the processed samples and to ensure proper sample storage before injection, reinjection reproducibility was evaluated. Accepted precision and accuracy batch samples were stored for a period of 24 h in an autosampler at 2 to 8 °C and the entire batch was re-injected for estimation of reinjection reproducibility.

Matrix Effect
Matrix effect for bexagliflozin was investigated at LQC and HQC. Six separate lots of blank dog plasma were extracted and the extract was spiked at LQC and HQC, separately. Analyte peak area in the samples was compared with neat standard solutions at the same theoretical concentrations (LQC and HQC). IS normalized factor and matrix factor were calculated.

Recovery
The recovery of bexagliflozin was assessed at three extracted levels (LQC, MQC, and HQC). The results were compared with the mean analyte response from post-extracted sample along with the internal standard at it working concentrations.

Stability
Stability of bexagliflozin in dog plasma was established at under following storage conditions: (1) Bench top stability (room temperature for 4 h) (2) Autosampler stability (autosampler 24 h), (3) Freeze-thaw stability (five cycles) (4) Long term stability (30 days at -70 °C).

RESULTS AND DISCUSSION

Design and Optimization of Method
A reliable and simple liquid LC/MS-MS method developed for identification of bexagliflozin in dog plasma. Among the ionization techniques tried APCI and ESI, ESI provided acceptable ionization and sensitivity for drug. Rolipram, a non-isotopically labeled compound, was selected as the internal standard for this study based on its similar chromatographic and ionization behaviour to bexagliflozin, as well as its stability and commercial availability. While stable isotope-labeled analogs are generally preferred for their superior ability to correct for matrix effects and variability in ionization, a deuterated IS for bexagliflozin was not readily available and is associated with significantly higher cost[12]. Although a deuterated IS may offer theoretical advantages, the low variability observed with rolipram, combined with acceptable accuracy and precision throughout validation supports the methods reliability for pharmacokinetic applications. In previously published methods, certain challenges were highlighted due to the structural and physicochemical nature of gliflozins[13-15]. Bexagliflozin is moderately nonpolar therefore can be adequately retained on C18 column. During method optimization, C18 stationary phases tried with the mobile phase consisting of variable amount acetonitrile, methanol and buffer (ammonium acetate and ammonium formate). Initially, a mobile phase consisting of equal volume of aqueous buffer and organic solvent was utilized to achieve optimal chromatographic separation. However, significant recovery was not achieved for bexagliflozin. Further optimization was achieved by changing the buffer concentrations to have better response and acceptable peak shape. It has been noticed that buffers with higher concentration (8-10 mM) improve peak shape and after rigorous optimization of various parameters, an optimal compromise among the retention time, resolution, analyte response, and peak symmetry was attained using mobile phase compromising methanol: acetonitrile (50:50) and 10 mM ammonium formate in milli-Q water in 90:10 v/v on BDS Hypersil C18 column. The retention time recorded for bexagliflozin was 1.86 within 3.5 minute run time.

Extraction procedures and preconditioning techniques plays crucial role in bioanalysis by eliminating unwanted interferences[16]. Sample clean-up is essential before

injection of samples on hyphenated technique. Over the year, various methods have been developed which are versatile, specific and selective. Selection of appropriate sample preparation approach is guided by the characteristics of the biological matrix and the physicochemical attributes of the analyte. Owing to their straightforward procedures and operational convenience, techniques such as liquid–liquid extraction[17], solid-phase extraction[18] and protein precipitation[19] continue to be widely employed for isolating drugs from biological samples. The selection of the solvent is critical and methanol as a precipitating agent was effective in getting good recoveries for bexagliflozin and rolipram.

System Suitability

System suitability tests were performed each day before analytical run at ULOQ and LLOQ level. Signal to rise (S/N) at LLOQ level for bexagliflozin in plasma was found to greater than 5. The % RSD of area ratio (analyte area/IS area) at ULOQ level for bexagliflozin was found less than 5.

Selectivity

Among six different lots of dog plasma, no interference observed at the retention time of analytes. Spiked LLOQ concentration was also found to be within ±20% of the nominal concentration.

Specificity

Specificity data demonstrated that the developed method was highly specific for bexagliflozin with showing no interference from any endogenous components at the retention times corresponding to bexagliflozin and rolipram.

Autosampler Carryover Test

The autosampler carryover experiment showed no carryover of bexagliflozin in the standard blank and reinjected the standard blank after injection of LLOQ and ULOQ.

Linearity

The calibration curve of bexagliflozin was linear and ranged between 10 to 1280 ng/mL which is demonstrated by equation Y= 0.00468 x + 0.00454 with a correlation coefficient greater than 0.99. All calibration point met the acceptance criteria. Linearity data are depicted (Table 1), chromatograms are presented (Figure 3, 4) and calibration curve is presented (Figure 5). $1/x^2$ weighting factor was applied to the regression model to minimize relative error across the concentration range. Also based on residuals plot, the residuals are scattered approximately randomly around zero and there is no trend in the spread of residuals with concentration.

Table 1: Linearity data of bexagliflozin

Nominal Concentrations (ng/mL)	Recovered Concentrations (ng/mL)	Accuracy (%)	Linear Regression Equation
10.02	9.98	99.6	Y= 0.00468 x + 0.00454 (r^2=0.997)
20.03	20.02	100	
40.06	40.73	102	
80.12	78.39	97.8	
160.24	168.42	106	
320.48	325.79	102	
640.95	620.91	96.0	
1281.91	1311.01	102	

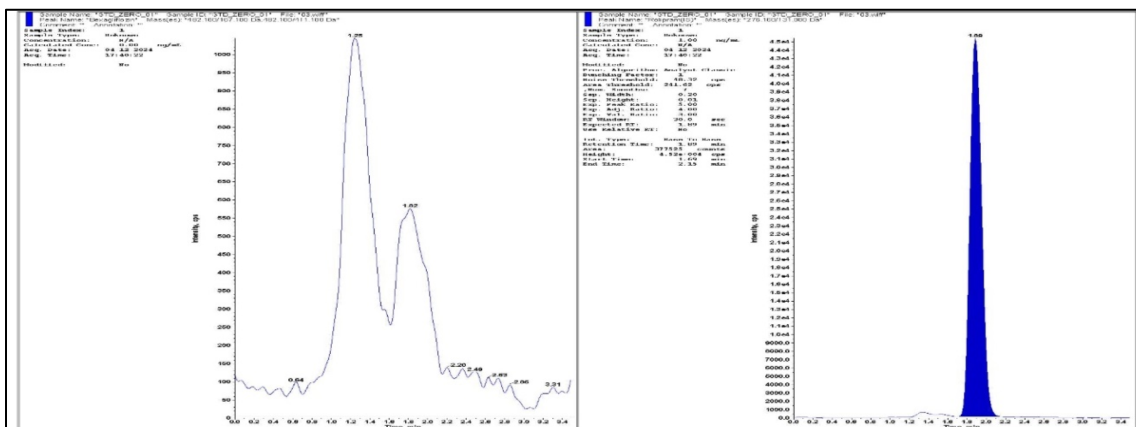

Figure 3: Chromatogram of standard zero sample

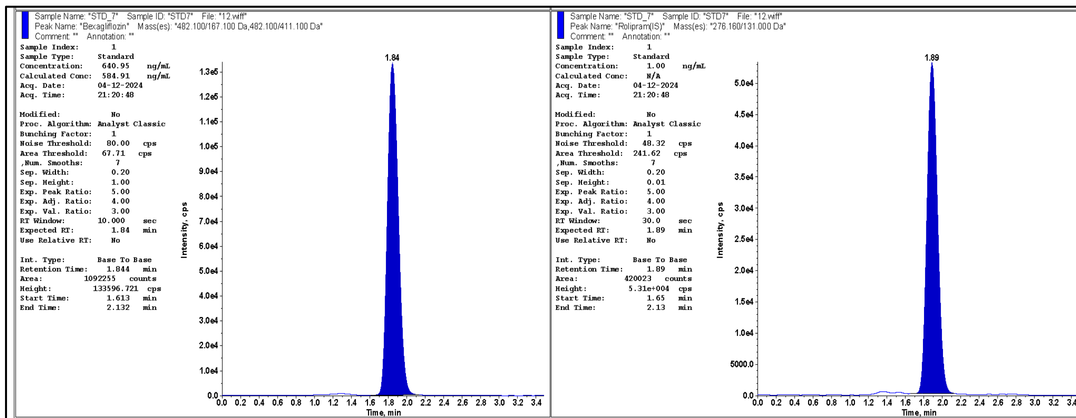

Figure 4: Chromatogram of calibration standard solution of bexagliflozin (160.24 ng/mL) with Rolipram (IS)

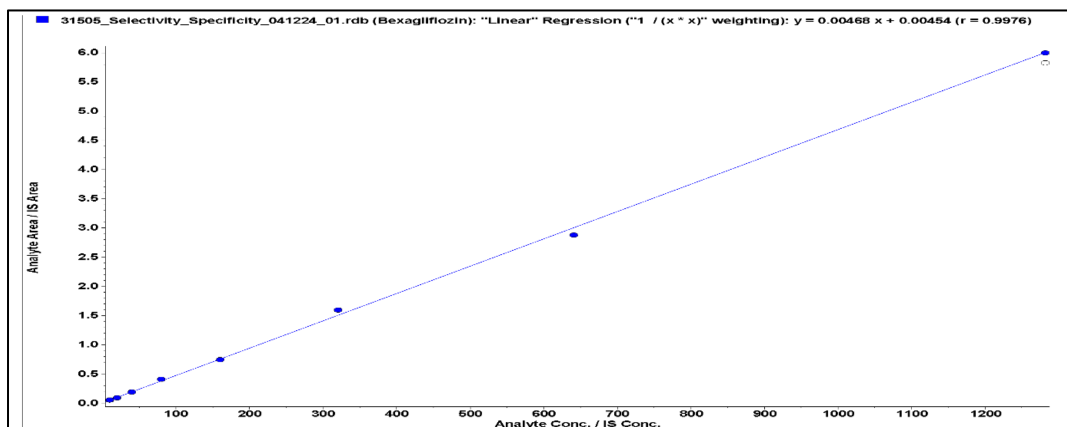

Figure 5: Calibration curve for bexagliflozin

LLOQ Determination

LLOQ of bexagliflоin in dog plasma determined to be 10.38 ng/mL. S/N ratio exceeded 5 with the recovered concentrations of the samples falling within ± 20% of the nominal values. Additionally, %CV for the samples was below 20%.

Precision and Accuracy

Precision and accuracy result for bexagliflozin detailed (Table 2) with data provided for each day. The percentage coefficient of variation (%CV) was found below 8.83 which is less than 15% of guideline limit. Accuracy levels ranged from 94.61% to 108.42% which is within ±15% of nominal concentration at all QC levels. These findings confirm that the method is robust and reliable for routine bioanalysis and potentially transferable across laboratories with minimal variation in performance.

Table 2: Data of precision and accuracy

Intervals	QC Level	Fortified Concentrations (ng/mL)	Recovered Concentrations (ng/mL)	% Accuracy	%CV
Day 1	LLOQ	10.38	10.74	103.47	6.70
	LQC	30.62	32.48	106.70	8.81
	MQC	577.14	599.48	103.87	8.83
	HQC	966.11	981.87	101.63	4.12
Day 2	LLOQ	10.38	10.54	101.54	5.57
	LQC	30.62	32.25	105.32	6.65
	MQC	577.14	596.98	103.43	8.29
	HQC	966.11	997.49	103.24	4.43
Day 3	LLOQ	10.38	9.82	94.61	5.09
	LQC	30.62	33.20	108.42	5.90
	MQC	577.14	600.11	103.98	3.42
	HQC	966.11	1007.92	104.32	5.69

Reinjection Reproducibility

Reinjection reproducibility was estimated over a 24 h period, showing accuracy between 96.41% and 106.12%, with precision values below 4.81.

Matrix Effect

Matrix-dependant signal suppression and enhancement is a common challenge in the quantification using liquid chromatography coupled with mass spectrometry. These effects can influence the quality of signals and exert deleterious impact on method parameters like LOD, LOQ, linearity, accuracy, precision etc. To understand these effects better and to minimize their influence, matrix match blanks have been used to validate analytical method parameters. The results obtained indicate that there were no significant matrix effects. The variation in the IS-normalized matrix factor for the analytes was calculated and are in between 0.91 and 1.09 indicating that the matrix components effect on the response of the analyte was insignificant.

Recovery

A simple protein precipitation method produced highly precise (%CV ≤2.1) and quantitative recovery of bexagliflozin (101.02–102.98%) across QC levels depicted (Table 3). The mean extraction recovery for the internal standard was around 101%.

Table 3: Results of % recovery

QC Level	Theoretical Concentration (ng/mL)	Recovered Concentrations (ng/mL)	%Recovery
LQC	30.62	31.01	101.28
MQC	577.14	582.99	101.02
HQC	966.11	994.88	102.98

Stability

Drug stability was performed at various experimental storage condition. Percentage recoveries for the stability samples ranged between 105.91% to 109.72% at defined stability conditions in plasma viz., 4 h bench top, 24 h autosampler, five cycle freeze-thaw and at -70 °C for 30 days long term. Detailed stability results for bexagliflozin in plasma is presented (Table 4).

Table 4: Results of stability

Condition	Recovery Level	Fortified Concentrations (ng/mL)	Recovered Concentrations (ng/mL)	% Accuracy	%CV
Benchtop Stability	LQC	30.62	32.57	106.37	2.91
	HQC	966.11	1060.02	109.72	2.87
Autosampler Stability	LQC	30.62	32.43	105.91	3.11
	HQC	966.11	1040.72	107.72	2.62
Freeze-thaw (5 cycles)	LQC	30.62	33.02	107.82	4.48
	HQC	966.11	1031.82	106.80	2.62
Frozen -70 °C	LQC	30.62	33.37	108.61	2.72
	HQC	966.11	1029.04	106.55	1.79

CONCLUSION

A robust LC–MS/MS analytical procedure incorporating a straightforward protein precipitation step was successfully established and validated to accurately quantify bexagliflozin in dog plasma. Significantly acceptable accuracy and precision were obtained over the linearity range. The method emphasizes simplicity, rapidity and provides information about control solution's stabilities. The proposed method has advantages with respect to simple extraction, procedure, processing method, sensitivity and reproducibility. The validation data showed that the method is precise, free from matrix effects and can be used for pharmacokinetics estimation.

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Abbreviations

LLOQC: Lower limit of Quantification, **IS**: Internal Standard, **ICH**: International Council on Harmonization, **MRM**: Multiple Reaction Monitoring, **ULOQ**: Upper Limit of Quantification, **LQC**: Low Quality Control, **QC:** Quality Control, **MQC**: Middle Quality Control, **HQC**: High Quality Control, **SGLT-2**: Sodium Glucose Co-transporter 2, **LC/MS-MS**: Liquid Chromatography and Mass Spectroscopy/Mass Spectroscopy, **ESI:** Electrospray Ionization, , **APCI**: Atmospheric Pressure Chemical Ionization

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