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Development and validation of a novel QuEChERS-Based HPLC-UV method for the determination of canagliflozin in human breast milk with measurement uncertainty assessment

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Abstract: A novel, simple, and reliable HPLC-UV method was developed and validated for the determination of canagliflozin in human breast milk. Sample preparation was performed using a QuEChERS based extraction procedure to overcome the complexity of the breast milk matrix. Chromatographic separation was achieved on a C18 column using isocratic elution with acetonitrile and water containing 0.1 % trifluoroacetic acid (70:30, v/v) at a flow rate of 1.2 mL/min, with UV detection at 290 nm. Canagliflozin was eluted at approximately 2.5 min within a total run time of 10 min. The method was fully validated in accordance with international bioanalytical guidelines. Linearity was demonstrated over the concentration range of 1–25 ng/mL with a correlation coefficient of r^2 = 0.9994. The limits of detection and quantification were 0.3 and 1.0 ng/mL, respectively. Mean relative and absolute recoveries were 99.53 % and 99.82 %, confirming the high efficiency of the QuEChERS extraction procedure. Intraday and interday precision values were below 1.27 % and 2.20 %, respectively. Robustness studies indicated that minor variations in chromatographic conditions did not significantly affect method performance. Stability studies confirmed that canagliflozin remained stable under various storage and analytical conditions. In addition, measurement uncertainty was evaluated using a bottom up approach, and the expanded uncertainty was calculated as 2.04 % at a 95 % confidence level. The proposed method offers a cost-effective and accessible alternative to mass spectrometric techniques and represents the first validated HPLC-UV method for the determination of canagliflozin in human breast milk using QuEChERS extraction.

Key words: canagliflozin, method development and validation, human breast milk, QuEChERS, uncertainty assessment

1. Introduction

Canagliflozin (*Fig.* 1) is a sodium glucose co-transporter 2 (SGLT-2) inhibitor widely prescribed for the treatment of type 2 diabetes mellitus.[1] By inhibiting renal glucose reabsorption, it increases urinary glucose

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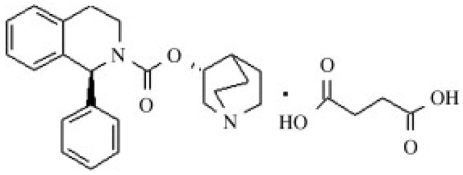


Fig. 1. Chemical structure of canagliflozin.

excretion, leading to effective glycemic control with additional benefits such as weight reduction and a low risk of hypoglycemia. Owing to its widespread clinical use, reliable analytical methods for the determination of canagliflozin in pharmaceutical formulations and biological matrices are of significant importance.[2,3]

To date, several analytical methods have been reported for the quantification of canagliflozin, including UV spectrophotometry,[4] HPTLC,[5] HPLC-UV/DAD,[6,7] and LC-MS/MS[8-10] techniques. These methods have primarily focused on bulk drug substances, dosage forms, and biological matrices such as human or animal and urine. Although LC-MS/MS methods offer high sensitivity and selectivity, they require sophisticated instrumentation and high operational costs, limiting their routine applicability in many laboratories.[11] In contrast, HPLC-UV remains a robust, cost-effective, and widely accessible technique for routine bioanalytical analysis.[12]

Breast milk is a complex biological matrix containing proteins, lipids, and carbonhydrates, and the presence of drug residues in breast milk is of particular concern due to potential infant exposure during lactation.[13] Despite the clinical relevance, no validated analytical method has yet been reported for the determination of canagliflozin in human breast milk. This represents a significant gap in the literature, especially considering the increasing prevalence of diabetes among women of childbearing age.[14]

Sample preparation is a critical step in bioanalytical method development, particularly for complex matrices such as breast milk. Conventional extraction techniques, including liquid-liquid extraction (LLE), are often time consuming, solvent intensive, and may suffer from limited selectivity.[15] The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach has emerged as an attractive alternative, offering efficient matrix clean up, reduced solvent consumption, and improved analyte recovery.[16] However, to the best of our knowledge, the application of QuEChERS extraction for the determination of canagliflozin in breast milk has not been previously reported.

In addition to method development and validation, the evaluation of measurement uncertainty has gained increasing importance in analytical chemistry to ensure the reliability and comparability of quantitative results. Nevertheless, uncertainty assessment is still included in routine HPLC-UV bioanalytical studies. Therefore, the aim of the present study was to develop and validate a novel, simple, and sensitive HPLC-UV method for the determination of canagliflozin in human breast milk using a QuEChERS-based sample preparation procedure. The method was fully validated according to international guidelines European Medicines Agency (EMA), and a comprehensive measurement uncertainty assessment (EURACHEM Guide) was performed. The proposed approach provides a practical and reliable alternative for routine analysis and contributes new analytical insight into the determination of canagliflozin in breast milk.

Although LC-MS/MS methods provide superior sensitivity and selectivity, their routine use in many analytical laboratories is limited by high acquisition costs, complex maintenance requirements, and the need for experienced operators. In contrast, HPLC-UV remains one of the most widely available analytical platforms worldwide. In the present study method selectivity was ensured through a combination of optimized chromatographic separation and an effecient QuEChERS-based clean-up strategy, which effectively removed endogenous breast milk interferences. The absence of co-eluting peaks at the retention time of canagliflozin, together with excellent recovery, precision, and robustness results, demonstrates that HPLC-UV can provide reliable quantitative performance even in a complex biological matrix when appropriate sample preparation is applied. Therefore, the novelty of this work lies not only in the application of HPLC-UV to human breast milk for first time, but also in demonstrating that a cost-effective and accessible

analytical platform can achieve performance characteristics suitable for routine lactation exposure assessment.

2. Experimental

2.1. Chemicals, solutions, and reagents

Canagliflozin reference standard (purity ≥99 %) was obtained from a certified pharmaceutical supplier (Shanghai Yingxuan Pharmaceutical Science & Technology Co Ltd. China). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA, ≥99 %) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), primary secondary amine (PSA), and C18 sorbent were used for QuEChERS extraction and clean-up. Ultrapure water was produced using a Milli-Q water purification system (Millipore, USA). Blank human breast milk samples were obtained from healthy volunteers and stored at -20 ºC until analysis.

2.2. Instrumentation and chromatographic conditions

Preliminary chromatographic studies were carried out to optimize the separation and quantification of canagliflozin. High performance liquid chromatography analysis was performed using a Shimadzu LC-20 series system (Kyoto, Japan) equipped with an LC-20AT solvent delivery pump, a SIL-20A autosampler, and SPD-20A UV-visible detector, and a CTO-10AC column oven.

During method optimization, different chromatographic parameters, including mobile phase composition, flow rate, and column characteristics, were systematically evaluated to obtain adequate peak shape and resolution. The optimized separation was achieved using ZORBAX Eclipse Plus C18 column (150 × 4.6 mm i.d., 5 µm particle size; Agilent Technologies, USA) operated at ambient temperature.

Isocratic elution was employed with a mobile phase consisting of acetonitrile and water (70:30, v/v), where the aqueous component was acidified with 0.1 % (v/v) trifluoroacetic acid. The mobile phase was delivered at a flow rate of 1.2 mL/min, and UV detection was performed at 290 nm, corresponding to the maximum absorbance of canagliflozin. The injection volume was 20 µL, and the total chromatographic run time was 10 min.

2.3. Preparation of standard and quality control solutions

A stock solution of canagliflozin was prepared at a concentration of 1 mg/mL by dissolving an accurately weighed amount of the reference standard in methanol. The stock solution was stored at 4 ºC and protected from light.

Working standard solutions were prepared daily by appropriate dilution of the stock solution with methanol. Calibration standards were prepared by spiking blank human breast milk samples to obtain final concentrations in the range of 1–25 ng/mL. Quality control (QC) samples were prepared independently at low, medium, and high concentration levels within the calibration range.

2.4. QuEChERS-based sample preparation

An aliquot of 1.0 mL human breast milk sample was transferred into a 15 mL polypropylene centrifuge tube. After spiking with appropriate volumes of canagliflozin standard or QC solutions, 4.0 mL of acetonitrile was added. The mixture was vortex mixed for 2 min to ensure efficient extraction.

Subsequently, a QuEChERS salt mixture consisting of 1.0 g $MgSO_4$ and 0.25 g NaCl was added. The tube was immediately shaken vigorously for 1 min and centrifuged at 5000 rpm for 5 min. For dispersive solid phase clean up, 2.0 mL of the upper acetonitrile layer was transferred into a clean centrifuge tube containing 50 mg PSA, 50 mg C18, and 150 g anhydrous $MgSO_4$. The mixture was vortexed for 1 min and centrifuged again at 5000 rpm for 5 min. the resulting supernatant was filtered through a 0.45 µm PTFE membrane filter, and 20 µL was injected into the HPLC system.

2.5. Method validation

The method was validated according to international

bioanalytical validation guidelines with respect to selectivity, linearity, accuracy, precision, recovery, and sensitivity. Calibration curves were constructed using six concentration levels at 1, 2, 5, 10, 15, and 25 ng/mL.

Intraday and interday accuracy and precision were evaluated using QC samples at three concentration levels and expressed as percent recovery and relative standard deviation (RSD%), respectively. The limits of detection (LOD) and quantification (LOQ) were estimated based on signal to noise ratios of 3 and 10, respectively.

2.6. Evaluation of measurement uncertainty

Measurement uncertainty was assessed using an analytical bottom up strategy by systematically identifying and quantifying the principal sources contributing to the overall variability of the results. The evaluated uncertainty components included the uncertainty associated with the purity of the reference standard ($u_{standard}$), the uncertainty arising from mass measurements during sample preparation ($u_{weighing}$), the uncertainty related to extraction recovery ($u_{recovery}$), and the uncertainty originating from calibration curve regression (u_{curve}). In addition, method repeatability was incorporated as an independent uncertainty contribution where applicable.

The combined standard uncertainty ($u_{combined}$) was calculated by applying the root sum of squares approach, assuming that the individual uncertainty components were uncorrelated, according to the following equation:

$$u_{Combined} = \sqrt{(u_{standard})^2 + (u_{weighing})^2 + (u_{recovery})^2 + (u_{curve})^2}$$

The expanded uncertainty (U) was subsequently obtained by multiplying the combined standard uncertainty by a coverage factor k = 2, which corresponds to an approximate confidence level of 95 %, as expressed below:

$$U = k \times u_{combined}$$

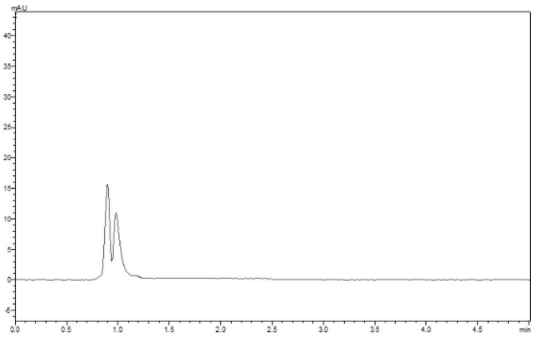
All uncertainty calculations were performed in accordance with the principles and recommendations described in the EURACHEM Guide[17] on measurement uncertainty and were further supported by relevant analytical chemistry literature.[18]

3. Results and Discussion

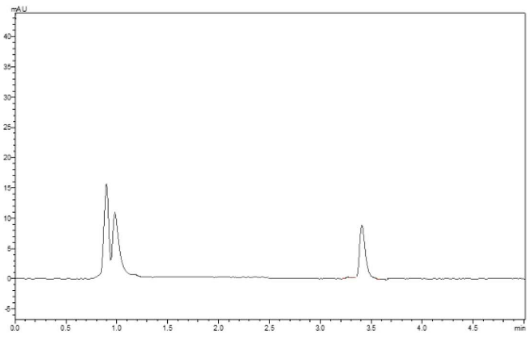
3.1. Chromatographic process

An isocratic chromatographic system was established to achieve reliable separation and quantification of canagliflozin in human breast milk. Representative chromatograms are presented in *Fig.* 2, illustrating (a) a blank breast milk sample and (b) a breast milk sample spiked with 10 ng/mL canagliflozin. No interfering peak originating from endogenous matrix components were observed at the retention time of the analyte.

Under the optimized chromatographic conditions, canagliflozin was eluted at a retention time of approximately 2.5 min. system suitability parameters, summarized in *Table* 1, confirmed the adequacy of the chromatographic system. The capacity factor



(a)



(b)

Fig. 2. (a) blank breast milk sample, (b) breast milk samples spiked with 10 ng/mL standard canagliflozin.

Table 1. Chromatographic system suitability parameters

Capacity factor*	Resolution*	HETP (μm)*	Tailing factor*	Asymmetry factor*
2.32	1.55	0.41	1.12	1.06

*Mean values of the parameters of all the points in the calibration study are mentioned

(2.32), resolution (1.55), theoretical plate height (HETP = 0.41 μm), tailing factor (1.12), and asymmetry factor (1.06) collectively indicate efficient column performance and satisfactory peak symmetry.

3.1.1. Optimization of chromatographic and QuEChERS extraction conditions

During method development, chromatographic parameters such as mobile phase composition, flow rate, and column temperature were evaluated to achieve adequate separation, acceptable retention time, and symmetrical peak shape. Different organic solvent ratios and operating conditions were tested, and the final chromatographic system was selected based on overall analytical performance.

The optimized method employed an isocratic mobile phase consisting of acetonitrile and acidified water (70:30, v/v, containing 0.1 % TFA) delivered at a flow rate of 1.2 mL/min. Under these conditions, canagliflozin exhibited sharp and symmetric peaks with a short retention time (2.5 min) and good reproducibility, making the system suitable for routine analysis.

For sample preparation, a QuEChERS based extraction procedure was optimized to overcome the challenges associated with the complex breast milk matrix. Acetonitrile was selected as the extraction solvent due to its effectiveness in protein precipitation and analyte partitioning. Subsequent dispersive solid phase clean up using PSA and C18 sorbents efficiently removed lipid and matrix interferences while maintaining high analyte recovery. Compared to conventional liquid-liquid extraction, the QuEChERS approach provided a faster, simpler, and more reproducible sample preparation strategy.

3.2. Validation of the method

The use of an internal standard is generally recommended in bioanalytical methods, particularly for mass spectrometric detection. However, in UV-based detection systems, the availability of structurally suitable and UV-active internal standards is often limited.

In this study, method robustness was ensured through highly reproducible extraction recovery (>99 %), low intra- and interday variability (RSD < 2.2 %), and stable chromatographic performance. The QuEChERS-based clean-up minimized matrix effects and injection-to-injection variability, recuding the practical necessity of an internal standard. Similar HPLC-UV bioanalytical studies have also reported acceptable validation performance without internal standardization when reproducibility and recovery criteria are met.

The develop method was validated in accordance with EMA bioanalytical method validation guidelines.[19] Based on the validation outcomes, the method exhibited high reproducibility, eliminating the necessity for the use of an internal standard.

3.2.1. Linearity and sensitivity

Linearity of the proposed method was evaluated by constructing calibration curves in human breast milk through plotting the chromatographic peak area versus canagliflozin concentration using a least squares linear regression model. Calibration standards were prepared at six concentration levels within the range of 1.0–25.0 ng/mL, and each level was analysed in six replicated. The resulting regression equation was: $y = 629.57x - 97.18$ with a high correlation coefficient of $r^2 = 0.9994$, indicating an excellent linear relationship between analyte concentration and detector response over the investigated range.

The sensitivity of the method was assessed by determining the limits of detection (LOD) and quantification (LOQ). These parameters were calculated according to the expression LOD or LOQ = k × SDa/b, where *k* corresponds to 3 for LOD and 10 for LOQ, SDa represents the standard deviation of the intercept, and b is the slope of the calibration curve. The analytical performance characteristics of the method, including the calibration range, regression equation, correlation coefficient, and sensitivity parameters,

Table 2. Analytical parameters of the method

Parameters	Method
Concentration range[a] (ng mL^{-1})	1.0-25.0
Regression equation[b]	y = 629.57× − 97.18
Intercept± SD	97.18 ± 11.66
Slope± SD	629.57 ± 88.21
Correlation coefficient (r^2)	0.9994
LOD (ng mL^{-1})	0.3
LOQ (ng mL^{-1})	1.0

[a]Average of six determinations
[b]$y = xC + b$ where C is the concentration in ng mL^{-1} and y is the peak area

are summarized in *Table* 2.

The obtained results demonstrate that the method provides a strong linear response acroos the tested concentration range and exhibits high sensitivity, with LOD and LOQ values of 0.3 ng/mL and 1.0 ng/mL, respectively. These findings confirm the suitability of the developed method for the accurate and reliable quantification of canagliflozin in human breast milk samples.

3.2.2. Accuracy, precision, and recovery

The accuracy and precision of the developed method were evaluated by analysing quality control (QC) samples prepared at three concentration levels representing the lower, middle, and upper regions of the calibration range. QC samples were prepared in human breast milk at 1.0, 10.0, and 25.0 ng/mL, and each concentration level was analysed in triplicate. Method accuracy was assessed basen on recovery values, while precision was expressed as relative standard deviation (RSD).

Absolute recovery was determined by comparing the peak areas obtained from breast milk samples spiked with canagliflozin prior to QuEChERS extraction with those obtained from non-extracted standard solutions at equivalent concentrations. The mean absolute recovery achieved using the QuEChERS-based extraction procedure was 99.82 %, indicating efficient analyte extraction from the complex breast milk matrix. Relative recovery, calculated by comparing the measured concentrations of spiked samples with the nominal added amounts using the calibration cuver, was found to be 99.53 %.

Intraday accuracy and precision were evaluated by analysing QC samples at all concentration levels within a single day, while interday performance was assessed over three consecutive days. The maximum intraday RSD value did not exceed 1.27 %, and the highest interday RSD was 2.20 %, demonstrating excellent repeatability and intermediate precision. The accuracy and precision results, summarized in *Table* 3, confirm that the proposed method provides reliable and reproducible quantification of canagliflozin in human breast milk.

3.2.3. Robustness

The robustness of the developed method was investigated to evaluate its reliability under small, intentional variations in analytical conditions. Quality control (QC) samples at three concentration levels were analysed in triplicate to assess the influence of these changes on method performance.

Deliberate modifications were applied to critical chromatographic parameters. The flow rate was varied

Table 3. Accuracy and precision of the method

Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean ± SD[1])	Recovery (%)	RSD of recovery	RSD of intra-day variation	RSD of inter-day variation
10	1	10.92 ± 0.03	99.27	0.55	1.27	2.20
	10	19.90 ± 0.01	99.50	0.48	1.19	2.05
	25	34.94 ± 0.01	99.82	0.34	1.10	1.97
Mean relative recovery = 99.53						

For each concentration n = 3

Table 4. Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	1.1	98.4	2.78
	1.3	97.6	3.01
Mobile phase composition (acetonitrile:aqueous phase)	75:25	98.1	2.90
	65:35	97.9	3.15
Column temperature	25	99.5	0.51
	35	99.7	0.46

n = 3 for all QC sample levels

from the optimized value 1.2 mL/min to 1.1 and 1.3 mL/min, the mobile phase composition was altered from 70:30 (v/v) acetonitrile-water to 65:35 to 75:25 (v/v), and the column temperature was adjusted from 30 ℃ to 25 ℃ and 35 ℃. these variations were introduced to examine the method's tolerance to minor deviations in operating conditions.

The obtained results demonstrated that none of the tested variations caused a significant effect on chromatographic performance. The highest RSD values observed were 3.01 % for changes in flow rate, 3.15 % for mobile phase composition, and 0.46 % for column temperature variation. The consistent peak response and satisfactory resolution obtained under all tested conditions confirm the robustness of the proposed method. A summary of the robustness results is presented in *Table* 4.

3.2.4. Stability

The stability of canagliflozin working standard solutions at quality control (QC) concentration levels was evaluated under various storage conditions to assess short term and long term stability. All stability experiments were performed in triplicate. The testes conditions included storage at room temperature in the dark for 24 h, storage in the autosampler at 4 ℃ for 24 h, and refrigerated storage at 4 ℃ for 30 days.

Under these conditions, the mean recovery values were found to be 98.6 %, 99.1 %, and 99.4 %, respectively. The relative standard deviation (RSD) values did not exceed 1.9 %, indicating minimal variability. These results demonstrate that canagliflozin standard solutions remain stable under the investigated storage conditions.

Matrix stability was further assessed using breast milk samples spiked with canagliflozin at three QC concentration levels (1.0, 10.0, and 25.0 ng/mL). Short time stability was evaluated by keeping spiked samples at room temperature for 6 h, while long term stability was assessed after storage at -20 ℃ for 30 days. In addition, freeze thaw stability was examined over three consecutive freeze thaw cycles.

All testes matrix stability conditions resulted in recoveries within the acceptable range of 95–105 % of the nominal concentrations, with RSD values below 3.0 %, indicating good stability of canagliflozin in human breast milk. Processed sample stability was evaluated by reanalysing extracted QC samples stored in the autosampler at 4 ℃ for 24 h. The obtained recoveries ranged from 98.8 % to 101.5%, with RSD values not exceeding 2.4 %. Overall, the stability studies confirm that canagliflozin is stable in both standard solutions and breast milk matrix under typical sample handling, storage, and analytical conditions.

3.3. Assessment of measurement uncertainty

Measurement uncertainty was evaluated using a bottom up approach and expressed as a percentage at a confidence level of 95 %. Individual uncertainty components related to reference standard purity, calibration, recovery, and repeatability were quantified and combined.

The combined standard uncertainty was calculated as 1.02, and the expanded uncertainty was determined to be 2.04 % (k = 2). The uncertainty budget, summarized in *Table* 5, indicated that calibration

Table 5. Uncertainity assessment for the developed method

Uncertainty (U) in %					
$U_{Standard}$	$U_{Calibration}$	$U_{Recovery}$	$U_{Repeatability}$	$U_{Combined}$	$U_{Expanded}$
0.483	0.832	0.318	0.124	1.022	2.044

contributed the most to the overall uncertainty, while sample weighing had a negligible effect. The low expanded uncertainty value confirms the reliability and traceability of the analytical results.

The uncertainity budget was constructed based on a bottom-up approach, allowing the relative contribution of each analytical step to be evaluated quantitatively. Calibration uncertainty was identified as the dominant contributor, primarily due to regression variability at low concentration levels. In contrast, weighing uncertainty contributed minimally to the overall uncertainty due to the use of calibrated analytical balances and gravimetric preparation procedures. The explicit identification of dominant uncertainty sources provides practical insight into which analytical parameters most strongly influence result reliability and offers guidance for future method optimization.

4. Conclusions

In the present study, a novel and robust HPLC-UV method combined with a QuEChERS based sample preparation procedure was successfully developed and validated for the determination of canagliflozin in human breast milk. The proposed method provides an efficient solution for the analysis of canagliflozin in a complex biological matrix, ensuring effective clean up, high recovery, and excellent reproducibility.

The method demonstrated satisfactory linearity over the concentration range of 1–25 ng/mL with a high correlation coefficient ($r^2 = 0.9994$). Low LOD (0.3 ng/mL) and LOQ (1.0 ng/mL) values confirmed the high sensitivity of the method. Accuracy and precision results were within acceptable limits, with mean relative and absolute recoveries of 99.53 % and 99.82 %, respectively, and intraday and interday RSD values not exceeding 1.27 % and 2.20 %. robustness and stability studies further verified the reliability of the analytical procedure under varied experimental conditions.

Furthermore, the inclusion of a comprehensive measurement uncertainty assessment strengthened the metrological reliability of the method, yielding an expanded uncertainty of 2.04 % at a 95 % confidence level. This aspect, which is rarely addressed in routine HPLC-UV bioanalytical studies, enhances the confidence in the reported quantitative results.

Overall, the proposed HPLC-UV-QuEChERS method represents a cost effective, sensitive, and reliable analytical approach for routine determination of canagliflozin in human breast milk. The method can be effectively applied in pharmacokinetic, lactation exposure, and quality control studies, offering a practical alternative to more complex and expensive mass spectrometric techniques.

Although the developed method was applied to spiked human breast milk samples, this approach is consistent with international bioanalytical validation guidelines when target analytes are not expected to be present in blank matrices. Canagliflozin is contraindicated or rarely prescribed during lactation, making the availability of naturally incurred samples extremely limited. Therefore, the validated method provides a reliable analytical tool that is readily applicable to real samples when clinical or pharmacokinetics investigations become available.

Conflict of Interest Statament

The authors declare no conflicts of interest/competing interests.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Bezmialem Vakıf University was approved by the Clinical Trials Ethics Committee (No: 2022/33 – E.63030).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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