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LC-MS/MS Method Development and Validation for Human Plasma Canagliflozin Determination

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Abstract

Hyperglycemia is treated with canagliflozin (CANA), for determination of CANA in human plasma, simple, accurate, precise, and reproducible RP-HPLC with mass spectrometry method was developed and validated via protein precipitation method. The average rate of CANA revival was 89.79 percent. There were no interfering endogenous components detected during the analyte's retention time. Bioavailability and bioequivalence investigations necessitating determination of CANA in human plasma can now make use of a straightforward and reproducible LC-MS-MS technique.

Keywords: Bioanalytical, LC-MS/MS, Canagliflozin, Protein precipitation, Validation.

Introduction

(*S*)-1, 5-anhydrocanagliflozin is the chemical formula.the methyl ester of 1-[3-[[5-(4-fluorophenyl)-2-thienyl]]-D-glucitol (Figure 1), a member of SGLT2 inhibitor family. Type-2 diabetes can be managed with its help. By blocking protein (SGLT2), canagliflozin prevents the kidneys from reabsorbing glucose and reduces the renal glucose threshold. Canagliflozin is effective for treating type-2 diabetes as either a monotherapy or in mixture with other medications. Its molecular weight is 444.52 and empirical formula is C24H25FO5S. It dissolves completely in DMSO and ethanol but almost hardly at all in water. Bioavailability is 65% and GI absorption is quick for this tablet, which is sold orally. It achieves peak concentrations in 1–2 hours and has a relative oral bioavailability of 65%. Canagliflozin is 99.99 percent protein-bound, most notably to albumin. Method validity was established using ICH-recommended analytical performance parameters. Canagliflozin, an inhibitor of SGLT2, is used to control hyperglycemia in patients with type 2 diabetes mellitus (DM). Increased glucose excretion in the urine and a decreased renal threshold for glucose (RTG) occur in a dose-dependent manner. In people who already have cardiovascular disease and type 2 diabetes, it is used to lessen the likelihood of significant cardiovascular events. Inhibiting SGLT2 is a non-insulin method of treating diabetes since it blocks glucose reabsorption, decreases the renal threshold of glucose, and enhances urine glucose excretion. Monotherapy with an SGLT2 inhibitor or combination therapy with other kinds of glucose-lowering medications is possible (1). White crystalline powder that dissolves completely in DMSO and ethanol but barely dissolves at all in water. It has a high bioavailability (65%) and is readily absorbed in the gastrointestinal (GI) tract. It has an approximate oral bioavailability of 65% and reaches peak concentrations in 1-2 hours. Albumin is the primary protein to which CANA binds (at a rate of 99%) (2).

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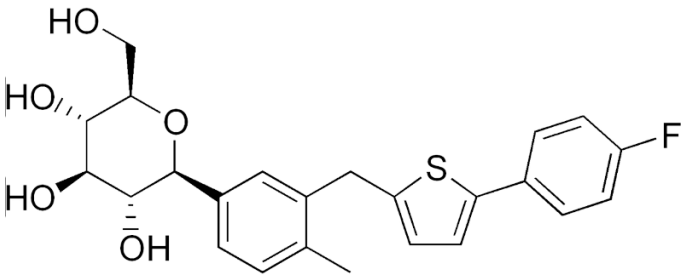

Figure 1: CANA structure

Experiments estimating CANA have been conducted using UV-HPLC alone, RP-HPLC in conjunction with HPLC in human plasma LCMSMS for formulation, and human plasma alone.

In the field of pharmacokinetics and clinical bioanalysis frequently emphasize the robustness of LC-MS/MS in achieving precise and reliable results, meeting regulatory standards for validation. This body of evidence underscores the method's versatility, rapid analysis, and structural elucidation capabilities, making it a well-established and preferred choice for pharmaceutical research involving compounds like canagliflozin.

Because of the complexity of the sample preparation procedures, the large amount of plasma required, the number of stages involved, and the low resolution of the peaks, the aforementioned methods fell short. With less time spent on sample prep and the added benefit of metabolite studies for pharmacokinetic analysis, IS was used to estimate CANA in the present investigation (3). LC-MS/MS is chosen for identifying canagliflozin in human plasma due to its high sensitivity, specificity, and quantitative capabilities. Its speed, selectivity, and compatibility with biological matrices make it suitable for high-throughput clinical studies. Regulatory acceptance, structural information provision, and versatility further contribute to its preference in pharmaceutical research and development.

Materials and methods

Canagliflozin was a gift from USV Private Limited, Acetonitrile and methanol of the LC-MS grade. Plasma from humans was gotten from the Malegaon Blood Bank in India. A Milipore XI5522050 with 0.45-micron membrane water purification device was used to get ultrapure water in-house. Additionally, everything else that was used was analytical grade and bought at regular chemistry shops (4).

Biological matrix employed in bioanalysis

Analytes are generally found in biological media like blood, plasma, urine, serum, feces, sweat, and so on. Protein precipitation was chosen as a straightforward technique over Liquid-Liquid Extraction and Solid Liquid Extraction following the selection of biological substrate (human plasma). To do this, you can make use of an acid that is both protein-precipitating and capable of dissolving the analyte. If you want to separate proteins, acetonitrile is the best solvent for you because it completely separates proteins into clumps that settle to the bottom after being centrifuged at high speed, leaving a clear liquid on top. Protein precipitation tries to reduce the amount of protein that gets into plasma and serum. This, in turn, lowers the damage that proteins, endogenous macromolecules, salts, small molecules, and metabolic leftovers do to HPLC columns (5-6).

Incubation condition and sample preparation

10 mg of CANA was dissolved in a 10 mL volumetric flask, and methanol was added until right amount was reached. This was used to make the primary standard stock solution. Once you have achieved a clear solution (main stock solution with 1000 PPM), stop shaking and stop ultrasonicating. Carefully remove 1 mL of the solution using the pipette and transfer it to a 10 mL volumetric flask. To the proper height, fill the flask with Methanol. Working standards were made by diluting secondary stock solution up to 10 mL with different solvents (20, 40, 100, 200, 400, 1000, and 2000 µL).

To test the drug, 250 µL of drug-free human plasma and 25 µL of test drug were mixed at 37± oC and stirred for 10 minutes. After the expected amount of time, the sample was prepared using chilled ACN

that contained an internal standard (25 µL). The mixture was then centrifuged at 1000 RPM for 10 minutes and then vertex for another 10 minutes. This causes the proteins to settle out and makes the supernatant solution clear. It was about 20 εL that was put into the HPLC machine (7).

Bioanalytical method validation

FDA-CDER recommendation for bioanalytical technique validation was used to make sure that the developed HPLC conditions were correct. Finding out the selection, recovery, precision, calibration curve, accuracy,and stability of analyte-spiked samples are all normal parts of developing and setting up a bioanalytical method (8).

Selectivity

Analytical selectivity refers to a technique's capacity to separate and quantify the analyte from background components in a sample. Selectivity requires at least six independent assessments of blank samples from relevant biological matrix (plasma, urine, or another matrix). Selectivity must be guaranteed at the LLOQ, and interference must be checked for in every blank sample. Endogenous matrix components, metabolites, and breakdown products, as well as associated medication and other exogenous xenobiotics, are all examples of substances that could potentially interfere with a biological matrix. Each analyte should be evaluated independently to rule out the possibility of interference if the method is designed to quantify multiple analytes.

Calibration/standard curve

The connection between measured analyte concentration and known instrument response is represented by a calibration (standard) curve (Figure 2). Each analyte in sample needs to have its own calibration curve. It is important to have a large enough sample size of standards when defining the correlation among concentration and response.

Lower limit of quantification (LLOQ): If following are accurate, LLOQ on calibration curve should be used.

a. Analyte reaction at LLOQ should be at minimum 5 times response relative to blank response.
b. A recognizable, separate, and repeatable analyte peak (response) with an accuracy of 80-120% and a precision of 20% is desirable.
c. Calibration curve

It is recommended to utilize the simplest model possible for describing the concentration-response connection. It is important to provide a rationale for the weights chosen and the sophisticated regression equation employed. It is recommended to utilize the simplest model possible for describing the concentration-response connection. It is important to provide a rationale for the weights chosen and the sophisticated regression equation employed. When creating a calibration curve, make sure these things are taken into account (9).

Precision

Accuracy of an analytical method is defined as consistency of results obtained from repeated measurements of a known analyte in identically diluted samples of a biological matrix. At least five concentration measurements should be made to determine accuracy. It is suggested that you use at least three concentrations that are in the usual range. There shouldn't be more than 15% of variation in accuracy at each dose level. The only exception is for the LLOQ, where there shouldn't be more than 20% of variation in accuracy. There are two types of precision measurements: those taken during an individual analytical run (intra-run precision) and those taken between runs (inter-run precision) and involving potentially different equipment, analysts, reagents, and laboratories (time-based precision) (10).

Accuracy

An analytical method's accuracy is defined as the degree to which method's mean test findings are in agreement with analyte's actual value. To make sure the results are accurate, duplicate tests of samples with known amounts of the analyte are used. The accuracy of a concentration measurement should be evaluated using at least five separate measurements. It is recommended to sample at least three concentrations within the estimated concentration range. The average value should be within a 15% margin of error, with the exception of LLOQ, where a maximum variance of 20% is permitted. The standard deviation from the mean is a measure of how close an estimate is to the true value being sought (11).

Recovery

Comparing detector response for analyte's

concentration in pure authentic standard to that obtained from adding and extracting the analyte from the biological matrix defines recovery. Within the bounds of its variability, recovery refers to the extraction effectiveness of an analytical procedure. Even if analyte recovery isn't perfect, it should be repeatable, accurate, and consistent. The same goes for internal standard recovery. The analytical results of low, medium, and high concentrations of extracted samples should be compared with those of 100% recovery standards that have not been extracted in order to conduct recovery studies (Table 1) (12).

Stability

Chemical qualities of the medication, matrix, and packaging all play a role in how long a drug will remain stable in a biological fluid. Freeze/thaw cycles, and the analytical method should all be evaluated for their effects on analyte stability. It is important to simulate real-world sample-handling and analysis settings when performing stability tests. To determine the stability of an analyte, a stock solution should be produced in a suitable solvent (13).

Precision

Prepare plasma samples spiked with canagliflozin at three different concentrations (low, medium, and high) within the calibration range. Analyze each sample in replicates (e.g., six replicates) within a single analytical run (intra-assay precision) and on different days (inter-assay precision). Calculate the percent coefficient of variation (%CV) for each concentration level. Acceptance Criteria: Intra-assay precision (%CV) ≤ 15%. Inter-assay precision (%CV) ≤ 15% (14).

Freeze and thaw stability

Three freeze/thaw cycles should be used to test stability of an analyte. At least three portions of the low and high amounts should be kept at temperature for 24 hours and then left to thaw at room temperature without any help. After the samples have been completely thawed, they should be frozen again for another 12-24 hours in the same circumstances. It is recommended to perform the freeze-thaw cycle twice more before analyzing the results. If an analyte isn't stable at the temperature it's supposed to be stored at, stability sample should be frozen at -700C for 3 rounds of freezing and thawing (15).

Short-term temperature stability

In order to conduct an accurate analysis, 3 aliquots of both low and high concentrations should be thawed at room temperature, where they will remain for between four and twenty-four hours (depending on how long the samples are expected to be kept in the research) (16).

Long-term stability

For a long-term stability test, elapsed time between sample collection and analysis should be longer than the storage time. To see how stable something is over time, you should keep at least three parts of it in the same conditions as the study examples. These parts should have different amounts of something. The samples ought to have enough to be checked three times. When you test for long-term stability, you should compare the concentrations of all the samples to the average of the values you found by going back and looking at the standards at the acceptable concentrations on the first day (17).

Stock solution stability

At room temperature for at least 6 hours, stock solutions of both medication and internal standard should be tested for stability. Stock solutions' stability should be documented if refrigeration or freezing is used to store them for the applicable time period. After the allotted amount of time has passed, the instrument response should be compared to that of freshly created solutions to determine how stable they are (18).

Post-preparative stability

It's important to measure how long processed samples stay stable after leaving the autosampler. Concentrations can be determined using the original calibration standards, and the stability of the drug and the internal standard can be checked over the course of the batch's planned run time using validation samples (19, 20).

Results and Discussion

Conditions utilized

Various combinations of acetonitrile, methanol, orthophosphoric acid, and ammonium acetate were systematically investigated in isocratic mode, with the aim of optimizing chromatographic conditions for the separation of analytes. Through these studies, it was observed that an optimal mobile phase composition was achieved using a phosphate buffer [60:40] in methanol, maintaining a pH of 2.5, and employing a flow rate of 1 mL/min. The chromatographic system operated at a working temperature of 25°C. The resultant chromatogram exhibited a Gaussian profile, and the observed narrower peak widths than anticipated indicated the achievement of ideal chromatographic conditions. (Figure 3) (21).

Development and optimization of mass conditions

We wanted to create a method that was both selective and sensitive, therefore we optimized the MS/MS settings accordingly. The mass spectrometer was primed to track the analytes of interest via direct infusion of standard solutions and optimal working conditions. In order to discover the parent and daughter ions and get maximal response for the analyte and the IS, positive ion mode tuning was applied. Each drug's MRM channel was selected based on its ability to generate as little or as much of a reaction from the other medications as possible. Prior to detection in multiple reactions monitoring (MRM) mode during monitoring at various transitions, three compounds were ionized using an ESI source. The signal response of MS/MS was maximized for the three analytes by optimizing gas flow, gas pressure, and gas temperature (Figure 4) (22).

Development of protein precipitation (PP) procedure

Several organic solvents were tested for use in the PP process. Acetonitrile as the PP solvent allowed for reasonable, clear, and reproducible recoveries while also streamlining the experiment and making the samples easier to handle. In developing and validating the LC-MS/MS method for canagliflozin in human plasma, ensuring selectivity against potential matrix interferences was pivotal. Chromatographic conditions were optimized for baseline separation, and matrix effects were systematically addressed by choosing suitable internal standards. Strategic adjustments to mobile phase composition and the application of selective extraction techniques further minimized matrix-related challenges. This comprehensive approach guarantees the method's reliability in accurately quantifying canagliflozin in the complex matrix of human plasma, meeting stringent bioanalytical validation requirements (23, 24).

Bio-analytical method validation

selectivity

Six different sources of drug-free human plasma with a concentration LLOQ (10 ng/mL) were used to test the selectivity of the analytical method. This was done to look for endogenous components that could interact with the analytes or IS. There was no major influence at any of the retention times for any of the plasma blank samples (25, 26).

Accuracy

The analysis of the data for canagliflozin determination reveals varying performance characteristics across different concentration levels. At a concentration of 10 ng/mL, the %RSD is within an acceptable range (12.36%), but the % recovery falls slightly below the anticipated value at 87.25%, indicating a potential need for further precision optimization. However, at higher concentrations (50, 500, and 1000 ng/mL), the %RSD values are all within acceptable limits (4.05%, 4.29%, and 5.18%, respectively). The % recovery for these concentrations also aligns well with expectations (90.81%, 91.07%, and 88.86%, respectively), indicating accurate quantification.

The average recovery across all concentrations is 89.50%, reflecting overall method reliability. Notably, the elevated standard deviation at 500 and 1000 ng/mL suggests potential room for improvement in precision at these concentrations. In conclusion, while the method demonstrates satisfactory accuracy and recovery, further optimization, particularly in terms of precision at lower concentrations, may be warranted to enhance overall method robustness and reliability for bioanalytical purposes (27, 28).

The validation of an LC-MS/MS method involves rigorous assessment of several key parameters to ensure its accuracy and reliability in meeting industry standards and legal requirements for bioanalytical method validation. Specificity, a crucial parameter, ensures that the method can accurately differentiate and quantify the analyte of interest in the presence of potential interfering substances. Sensitivity gauges the method's ability to detect low concentrations of the analyte, often vital for pharmacokinetic studies. Accuracy is validated by comparing the measured values to the true values, affirming the method's reliability in quantification. Precision, encompassing both repeatability and intermediate precision, underscores the method's consistency in reproducing results under varying conditions. These validation parameters collectively contribute to the method's robustness and adherence to regulatory guidelines, instilling confidence in the accuracy and dependability of the LC-MS/MS method for bioanalytical applications (29, 30).

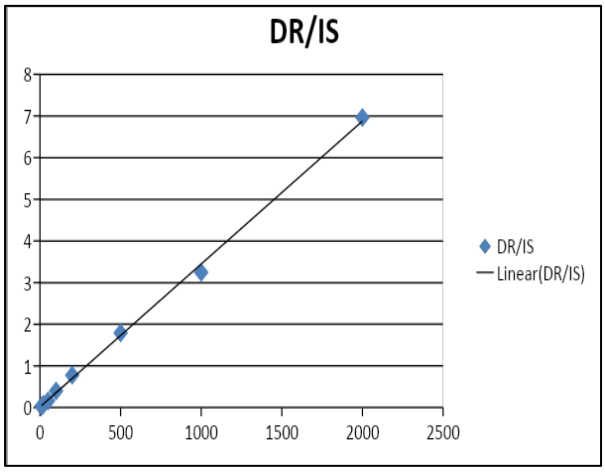

Figure 2: Calibration Curve for CANA

Table 1: Recovery studies of the method to determine the accuracy

Conc ng/mL	Average DR/IS	SD	%RSD	% recovery	Avg recovery
10	0.03376	1.07844	12.35999	87.25225225	89.49898
50	0.16661	1.84054	4.05345	90.81338144	
500	0.12087	19.54703	4.29256	91.07401713	
1000	0.38522	46.04034	5.18144	88.85625338	

Table 2: Stability study for CANA in human plasma

Conc ng/mL	Short term Room temp 24hrs			Freez Thaw Stability			Long Term Stability		
10	0.035718	0.004405	12.3327	0.035718	0.004405	12.3327	0.035718	0.004405	12.3327
50	0.171056	0.013155	7.690352	0.171056	0.013155	7.690352	0.171056	0.013155	7.690352
500	0.123655	0.008359	6.759648	0.123655	0.008359	6.759648	0.123655	0.008359	6.759648
1000	0.391944	0.027735	7.076241	0.391944	0.027735	7.076241	0.391944	0.027735	7.076241

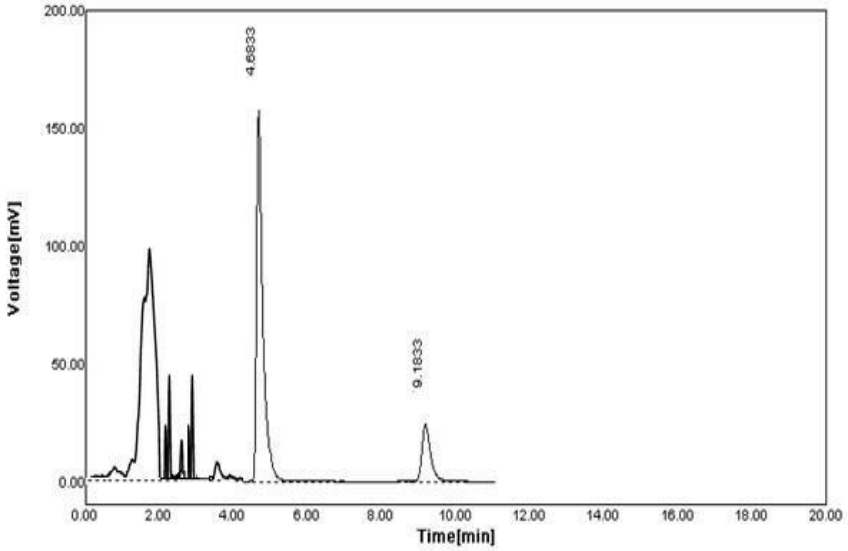

Figure 3: Typical chromatogram of CANA and IS with retention time 4.68 and 9.18 minutes respectively

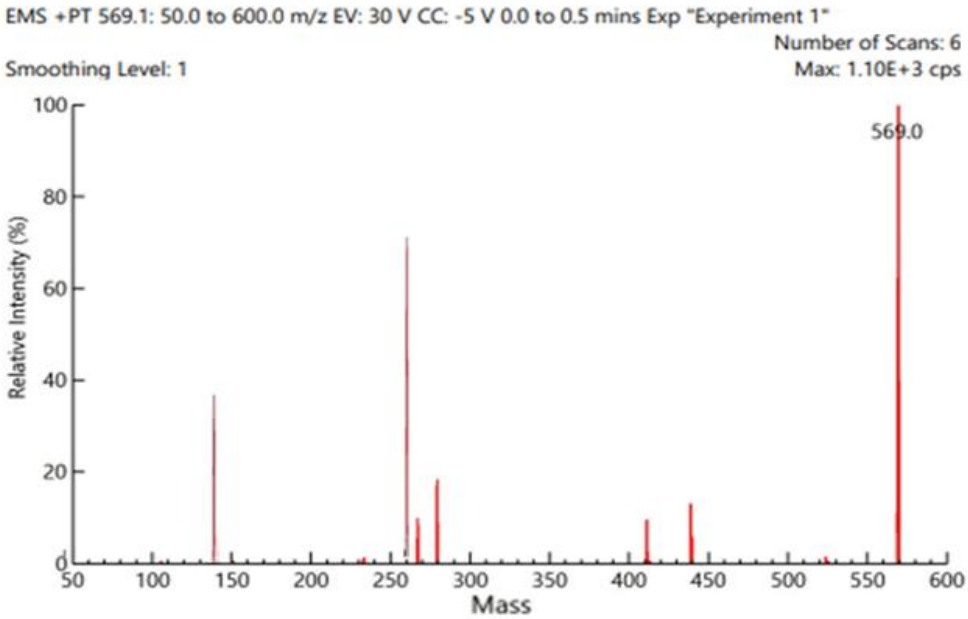

Figure 4: Representative ESI Mass Spectra Scans for the daughter ion of CANA

Stability

We looked at LLOQ to HQC, which contains the standard range points for the analyte following various settings, to determine the stability of analytes in human plasma. Four copies were stored at 25 degrees Celsius for short-term stability. After 6 hours, the samples were processed and examined and the results were compared to the amounts that were used at the start. Four duplicates of both the low- and high-QC plasma samples were frozen at -70°C 15 for long-term storage. There was a comparison between the final amounts and the initial amounts used after 30 days of processing and analysis of the samples. Samples of both low and high quality control were frozen and thawed three times for testing. The samples were examined after three cycles, and the results were compared to the

assumed normal concentrations. After storing drug and IS stock solutions at room temperature (25°C) for around 6 hours, we compared them to freshly prepared solutions to determine their stability. Tables 2 display results of stability test, which demonstrate satisfactory stability (31-35).

Pharmacokinetic application

Tandem Mass spectroscopy was added to the RP-HPLC method that was created. Human Plasma has become active for the analytes after NADPH enzymes were added. The fact that there are two daughter peaks at m/z 438.63 and m/z 279.14 shows that the suggested method can be used for *in vitro* studies that need accurate metabolite measurement. By taking out the molecules and studying how they work in human plasma, more toxicological research can be done (36-50).

Conclusion

We have created, improved, and confirmed an LC-MS/MS method that can accurately and selectively measure Canagliflozin in human plasma. Canagliflozin is a strong angiotensin-II receptor blocker drug that is used to treat high blood pressure. The extraction process was quick and easy, and the results were reproducible. Isocratic runoff and a short run time make analysis possible quickly. So, the test makes it possible and easy to check on their therapy when it's needed quickly and economically.

Abbreviations

LC: Liquid Chromatography, MS: Mass Spectroscopy, CANA: Canagliflozin, LLOQ: Lower limit of Quantification, *HPLC:* High-performance liquid chromatography, *LOD:* Limit of detection, *RP:* Reverse phase, *QC:* Quality control, *HQC:* Higher quality control, DM: Diabetes Mellitus, RTG: Renal threshold for glucose, SGLT 2: Sodium-Glucose Transport Protein 2, IS: Internal Standard.

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Author contributions

HT, SD – Writing, SA, AB – Revisions, KW – Proofreading.

Conflict of interest

No conflict of interest.

Ethics approval

Not applicable.

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