



Analysis of Canagliflozin in Rat Plasma After Oral Administration by Liquid Chromatographic as (Pharmacokinetic Study)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The research work aims to develop a bioanalytical method using liquid chromatography and validated for the determination of canagliflozin by using an internal standard. Department of Pharmaceutical Chemistry, Smt. Kishoritai Bhoyar College of Pharmacy, New Kamptee, Nagpur (MS). Isocratic chromatography separation was achieved on an LC system with PDA detector on an ACE C18 (150mm× 4.6mm × 5µm) column using a mobile phase composition of acetonitrile: ammonium acetate buffer in the ration of 50:50 v/v (pH 4.5), orthophosphoric acid is used to adjust pH of mobile phase and the flow rate at 1.0ml/ min. and estimation was carried out at 291 nm. The retention time of a drug was 4.633 minutes. The method was validated for several parameters (specificity, linearity, precision, and accuracy) and also successfully applied for the pharmacokinetic in female rats. Calibration plot was linear ($r^2 > 0.9973$) over the concentration range of 5-30 µg/ml for canagliflozin. The high recovery and low relative standard deviation (%RSD) confirm the suitability of the method. The result of Limit of Detection (LOD) and Limit of Quantitation (LOQ) were found to be 0.1099 µg/ml and 0.3331 µg/ml, respectively. The new RP-HPLC method can be conveniently adapted for examining canagliflozin concentration in rat plasma after oral administration.

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Keywords: Canagliflozin; high-performance liquid chromatography; method validation; pharmacokinetic assessment.

1. INTRODUCTION

Canagliflozin (2S, 3R, 4R, 5S, 6R)-2-{3-[5-[4-Fluoro-phenyl)-thiophen-2-methyls]-4-methyl-phenyl}-6-Hydroxymethyl-tetrahydro-pyran-3, 4, 5-triol is an anti-diabetic drug. The category of canagliflozin is Sodium-glucose co-transporter 2 [SGLT-2] inhibitor. Hyperglycemia is used for the treatment of type-II diabetes mellitus [1,2]. Canagliflozin is used to decrease excessive risk of cardiovascular events occurred to the diabetic mellitus patient. From the renal tubular lumen SGLT-2 reabsorb filtered glucose. The renal threshold increases glucose excretion via urine and it is reduced by using canagliflozin and also used to inhibit reabsorption of filtered glucose [3].

Canagliflozin is a white to off white powder. It is basic compound with PKa 12.57. It is soluble in Ethanol, Methanol, Tetrahydrofuran acetone. Insoluble in aqueous media and belongs to phenolic glycosides category. It is a well characterized small molecule with molecular formula $C_{24}H_{25}FO_5S$ and molecular weight is 444.52 g/mole [4,5].

Several methods reported for the HPTLC, UV-Visible Spectrophotometric [6,7,8]. Through the literature Survey it was confirmed that there is no internal standard method has been reported for canagliflozin drug. So, the main purpose of the present study is to investigate to develop new internal standard method which is more precise, specific, sensitive and accurate and also aimed at pharmacokinetic study by using female rat plasma. According International Conference on Harmonization (ICH) guidance (ICH 2000) and USP 29 (United State Pharmacopoeia,2005), validation of drug is done by using the finalized optimized method. Dapagliflozin is used as internal standard. The chemical structure of canagliflozin is shown in the Fig. 1[4].

2. EXPERIMENTAL

2.1 Chemical and Reagents

Canagliflozin was a gift samples from Indoco Remedies Limited. Ammonium acetate o-phosphoric acid was supplied by Merck Specialties Private Limited. Tetrahydrofuran was supplied by LOBA Chemie Private Limited. HPLC grade acetonitrile was purchased from Merck Life Science Private Limited. Double distilled water was used throughout the work. Solvents used for HPLC were of HPLC grade and all other chemicals and reagents were of analytical grade.

2.2 Instrumentation

The HPLC Shimadzu 1100 series chromatograph equipped with isocratic pump L-10ADVP series PDA detector with Rheodyne injector. Sample injected with Rheodyne injector with a 20 μ L Loop. The chromatographic analysis was carried out by LC solution software for data acquisition and processing.

2.3 Chromatographic Parameters

The analytical column was ACE C_{18} (150mm \times 4.6mm \times 5 μ m) column used for canagliflozin drug. The composition of mobile phase was acetonitrile and ammonium acetate buffer (pH 4.5) in the ratio of 50:50, respectively. The prepared mobile phase was filtered through a 0.45 μ m membrane filter paper and sonicated for 10 minutes. Chromatographic analysis was performed at a flow rate of 1.0 mL/min. The optimized wavelength selected was 291 nm, which represents the wavelength of maximum response for canagliflozin. Sample analyzed by using PDA detector covering the range of 200-400nm.

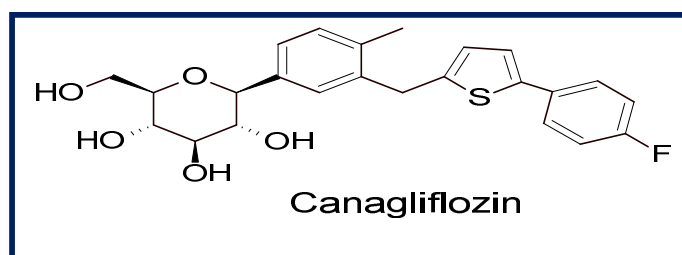


Fig. 1. Chemical structure of canagliflozin

2.4 Preparation of Calibration Curve of Canagliflozin

A working stock solution of Canagliflozin (100 µg/mL) was prepared by transferring 0.0025g of drug in 10 ml methanol and sonicated for 10 minutes and volume made up to mark with methanol (25ml). Further, multiple-point calibration curve (CC) was prepared by serial dilution of working stock solution in the range of 0.5-2.5 ml at the 0.5 difference and transferred to 10 ml volumetric flask and mixed with a sufficient quantity and volume made upto mark with diluent to obtained the concentration range 5-30 µg/ml for canagliflozin. The absorbance for each solution was recorded spectrophotometrically at 291 nm.

2.5 Bioanalytical Method Validation Study

As per ICH and FDA guidelines, the optimized method was validated using canagliflozin with respect to the following parameters: accuracy, precision, LOD, LOQ, specificity and system suitability [9,10].

2.5.1 Study of system suitability parameter

The system suitability parameter was carried out after equilibration of column with mobile phase, six replicate injections of 20 µl solutions were injected through the manual injection and chromatographed. From chromatograms, the peak response (i.e., peak area) were recorded and %RSD, SD and correlation coefficient was calculated.

2.5.2 Linearity

The linearity of an analytical method is to elicit test results that are proportional to the concentration of analyte in sample within a given range or proportional by means of well-defined mathematical transformation. The working standard stock solution was prepared by weighing quantity about 2.5mg was transferred to 25 ml volumetric flask and then dissolved in 10 ml of methanol, sonicate for 10 minute and volume made up to mark with methanol. Aliquots of working standard stock solution were diluted in range of 0.5-3.0 ml canagliflozin and 2.0 ml of dapagliflozin in 10.0 ml of volumetric flask with methanol and volume was made up to mark with diluent to obtained concentration ranging from 5-

30 µg/ml of canagliflozin and 20 µg/ml dapagliflozin.

2.5.3 Specificity/selectivity

Specificity is important features of HPLC is known for differentiating the analyte and the other components in the complex mixture [11,12]. Specificity ensure that the co-former did not affect the determination of the drug and the co-former is used for the preparation of co-crystals. Selectivity and specificity were validated by analyzing the blank plasma from female rats to test interference at the analyte retention time. The proposed procedure was used for testing each blank plasma sample. Further, the chromatograms were recorded to detect any peak present at the retention time (RT) of canagliflozin and internal standard compared with the result of plasma sample.

2.5.4 System precision

Precision of any analytical method is defined as Standard Deviation (SD) and Relative Standard Deviation (%RSD) of series of measurement. Precision was divided into interday precision and intraday precision (Repeatability). Precision of estimation by proposed method was ascertained by replicate analysis of homogeneous samples of standard canagliflozin with constant concentration of internal standard.

2.5.5 Accuracy:

Accuracy of optimized method was finding out by using standard addition method. Canagliflozin drug was added at three different levels in solution form with mixed amount of internal std. in series 50 ml volumetric flask and volume made upto the mark with mobile phase. Aliquot 0.5 ml from the above solution and further diluted upto 10 ml with mobile phase.

The accuracy was determined by comparing the assayed concentration with the nominal concentration.

Accuracy is calculated as percentage of recovery using the following equation:

$$\% \text{Recovery} = \frac{\text{Total amount estimated} - \text{Label Claim} \times 100}{\text{Amount of standard drug added}}$$

2.5.6 LOD and LOQ:

The standard deviation of y-intercept and slope of calibration curves were used to calculate the LOD and LOQ for all the drugs using following formulae.

$$\text{LOQ} = 3 (\sigma)/S$$

$$\text{LOQ} = 10 (\sigma)/S$$

Where,

σ = Standard deviation (SD) of response

S = Slope of the calibration curve

2.6 Pharmacokinetic Study in Rats

Female (200-250g) Wistar rats were housed in experimental groups (four animals) for pharmacokinetic study. According to the guidelines, the care and use of laboratory animals was done. Before experimentation, over a week the animals were adjusted to laboratory conditions and feed with standard rat diet. Before the experiments, the rats were housed in a temperature and humidity-controlled room ($23 \pm 3^\circ\text{C}$; air humidity, $55 \pm 5\%$ RH) and under controlled conditions of a light: dark (12:12 h) cycle with free access to water. The twelve group was selected by random division of female rats and was fasted for 12 hours with free access to water before the experiments. They receive oral formulations (canagliflozin std. S1, S2, S3, S5, S6, S7, S8, S10, S11, S13, blank) at a dose equivalent to 3mg/kg of canagliflozin. Blood sample (0.5ml) were collected from the eye vein at 2, 4, 6, 8 and 24 hours after treatment. Then the plasma sample were separated by centrifugation at 15,000 rpm for 15 minutes and stored at -20 until analysis.

2.6.1 Sample preparation

Frozen blood samples were thawed at room temperature and prepared for analysis. Pipette out 0.2 ml of blood and then added 100 μl methanol and 1 ml of Tetrahydrofuran and the mixture were vortex-mixed for 30sec. To extract canagliflozin after centrifugation at 15000 rpm for 15 minutes, the precipitate containing protein was discarded, the supernatant was collected and add 20 μl methanol containing 20 μg dapagliflozin as an internal standard and evaporated to dryness. The residue obtained was re-suspended in 100 μl mobile phase and vortex-mixed for 30 sec. and the 20 μl solution

was injected to HPLC system for analysis. Canagliflozin was detected at a wavelength of 291 nm.

The same procedure used for each sample,

Concentration calculated using a following formula...

$$\frac{\text{Area of internal std.}}{\text{Area of std. API/sample}} = F \frac{\text{Conc. of internal std.}}{\text{Conc. of std. API/sample}}$$

3. RESULTS AND DISCUSSION

3.1 Saturation Solubility of Canagliflozin:

The solubility of canagliflozin was determined at room temperature. An excess amount of sample (10mg) was added to 25 ml of beaker containing 5 ml of water. The sample was rotated on magnetic stirrer at 30 rpm for 24hr. after that solution was filtered rapidly through Whatman filter paper no. 41 and pipette out 1 ml of solution from filtrate and the dilution was done using 10 ml of methanol. The filtrate solution analyzed using UV spectrophotometer at 291 nm. Solubility was calculated using

$$CU = \frac{CS}{AS} \times AU \times \text{dil. factor}$$

Result of saturation solubility are 2.9088 $\mu\text{g/ml}$.

3.2 Method for HPLC-UV Conditions

A relevant wavelength of drug is important for better sensitivity. The strong UV absorption and spectra was recorded at the wavelength 291 nm. Hence the detection wavelength was set as 291 nm. It was necessary to use the internal standard (IS) in the technique and HPLC method to compensate for variations, efficiency and analytical errors. In the study, dapagliflozin was used as an internal standard (IS) because the chemical structure of dapagliflozin is somewhat similar to canagliflozin and its behavioral characteristics and physical and chemical properties confirm to the chemical requirement for internal standard in HPLC. In addition, commercial availability of dapagliflozin is highly pure and stable and it never react with the sample or mobile phase. The mobile phase was optimized through several trials to achieve finer resolution and symmetric peak shapes for both

the analyte and the internal standard, as well as a short run time. The mobile phase composition Ammonium acetate buffer (pH 4.5) and acetonitrile was selected in the ratio of 50:50 v/v and stationary phase was used in this study was ACE C₁₈ (150×4.6×5μ) column with a flow rate of 1.0 ml/min to produced better peak shapes and retention time of canagliflozin standard was at 4.8 min.

3.3 System Suitability Study

The system suitability parameters were shown in Table 1. The parameters are peak area, retention time, number of theoretical plate and

tailing factor were calculated. The USP theoretical plate was found to be less than 6000 and tailing factor was found 1.832.

Linearity: By preparing the series of dilution of a standard stock solution the linearity illustrated on the test sample directly. The identified response of solution was found to be linear over the concentration range 5 to 30 μg/ml. The regression equation for the graph is $y = 56440x + 92309$ and the correlation coefficient (R^2) was found to be 0.9973 showing better correlation between the area and the concentration and it is shown in Fig. 2. The Area Under Curve (AUC) of canagliflozin are given in Table 2

Table 1. Evaluation data of system suitability test

Sr. no	Standard weight taken (mg)	AUC of CANA (mV)
1	~10.0 mg	651093
2		664981
3		665405
4		653780
5		663374
6		652685
	Mean	658559
	%RSD	1.01
	Theoretical plate/column	56920.83
	Retention time	4.633 ± 0.1
	Tailing factor	1.832

Table 2. Observation of linearity response

Sr. no	Dapagliflozin conc.(μg/ml)	Canagliflozin conc.(μg/ml)	AUC (mV) dapagliflozin	AUC (mV) canagliflozin
1	20	5	155747	347536
2	20	10	155747	683112
3	20	15	155747	952295
4	20	20	155747	1199195
5	20	25	155747	1536238
6	20	30	155747	1761683

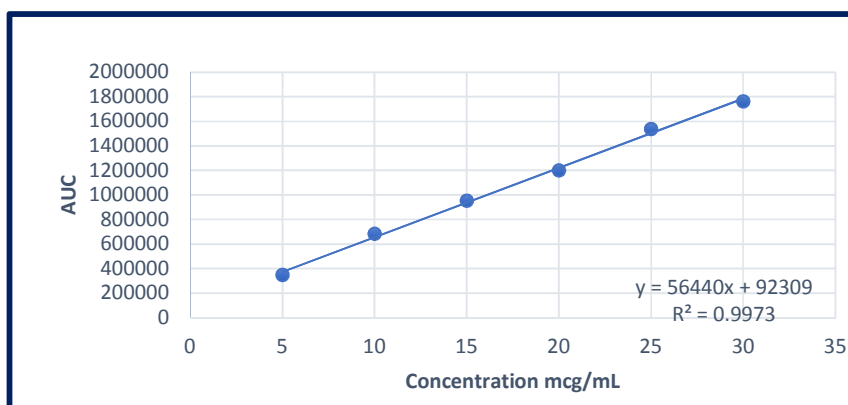


Fig. 2. Calibration curve of canagliflozin

Specificity: Specificity is important features of HPLC is known for differentiating the analyte and the other components in the complex mixture [11]. It was performed by using blank plasma detection, peak purity with pure standard compounds. Blank plasma had no interference, when canagliflozin and the Internal standard were eluted. The separation of blank plasma with internal standard is shown in the Fig. 3. At optimized conditions, the retention time of Canagliflozin at 4.758 and Internal Standard (IS) at 5.709. the separation of canagliflozin and IS was completed within 5 minutes and it is given in Fig. 4.

Precision: Precision of any analytical method is described as SD an RSD of series of

measurement. Precision evaluation was carried out by proposed method as replicate analysis of homogeneous samples of std. canagliflozin with constant concentration of internal standard were injected in the HPLC and SD and RSD were calculated. The percentage relative standard (%RSD) of the area of canagliflozin during intraday study and interday was found to be less than 0.11 and 0.53, which indicate a better precision of the method and the result was shown in Table 3. Intraday and inter day precision (% precision) of the method are within the acceptance limits to meet the guidelines for bioanalytical method validation which is considered to be $\leq 15\%$.

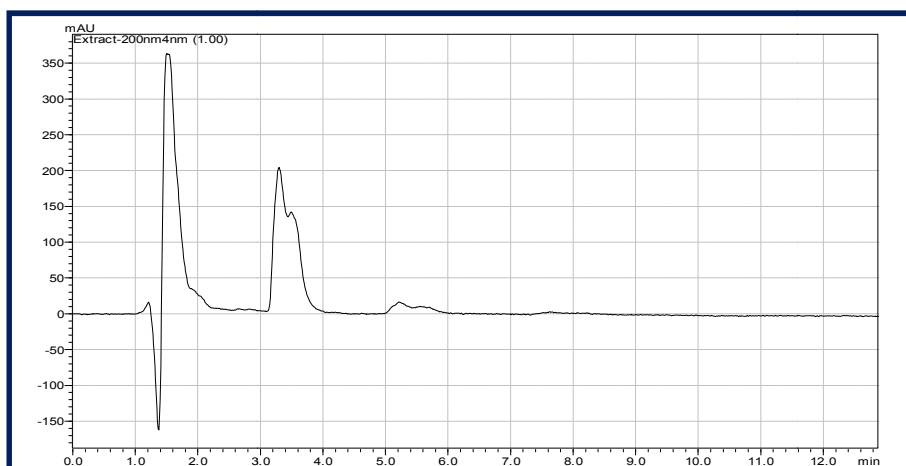


Fig. 3. Chromatogram of blank plasma with internal standard

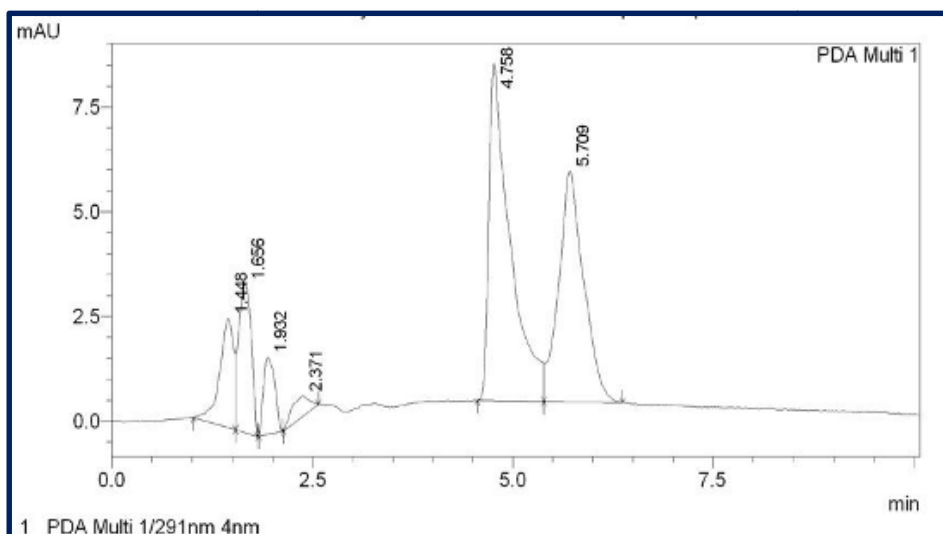


Fig. 4. Chromatogram of blank plasma with internal standard and drug canagliflozin

Accuracy: The quantitative recovery study was carried out at three concentration levels and the % accuracy values are given in Table 4. The percentage recovery of canagliflozin achieved ranged from 107, 108 and 101 % for three concentration levels respectively. The acceptable criteria for the mean value should not be deviated more than $\pm 20\%$.

Limit of Detection and Limit of Quantitation: The LOD and LOQ of canagliflozin was evaluated by calibration curve which include signal-to-noise ratio, use of standard deviation of the response and slope of the calibration curve. Concentrations of Limit of Detection (LOD) and Limit of Quantitation (LOQ) were found to be 0.1099 $\mu\text{g/ml}$ and 0.3331 $\mu\text{g/ml}$, respectively.

Application of the assay: The validated method was applied to investigate the content of prepared canagliflozin co-crystals in vivo, after administered orally to female rats. Pharmacokinetic studies in rats were carried out to evaluate the oral absorption of canagliflozin. The pharmacokinetic parameter like AUC_t , AUC_i , C_{max} , T_{max} , $t_{1/2}$, were calculated using pharmacokinetic software PK solver 2. The main pharmacokinetic parameter is listed in Table 5. In the study, oral administration of canagliflozin was resulted as a sharp C_{max} of 10.551 $\mu\text{g/ml}$ at 6 h and after that the plasma concentration reduced rapidly by indicating a fast absorption of canagliflozin. The area under the concentration versus time curve was 176.6781 $\mu\text{g/ml} \cdot \text{h}$. The

Table 3. Result of precision study

Sr.no.	Concentration (CANA) in $\mu\text{g/ml}$	Observed Concentration in $\mu\text{g/ml}$	AUC (mV)
INTRADAY			
1		30.09	1766545
2	30	30.06	1764789
3		30.01	1761683
Mean			1764339
SD			2010.246
%RSD			0.11
INTER DAY			
1		28.28	1660610
2	30	27.92	1639401
3		28.18	1654315
Mean			1651442
SD			8893.669
%RSD			0.53

Table 4. Result of accuracy study

Sr. no.	Observed concentration in $\mu\text{g/ml}$	% Accuracy
1	1.28	107.00
2	2.70	108.00
3	5.05	101.00

Table 5. Pharmacokinetic parameters of canagliflozin

Pharmacokinetic Parameters	Canagliflozin
C_{max} $\mu\text{g/ml}$	10.551
T_{max} (h)	6
$T_{1/2}$ (h)	50.2203
AUC_{0-24} $\mu\text{g/ml}$	176.6781
AUC_i $\mu\text{g/ml}$	745.6937
%Relative bioavailability	-

results proved that the suitability of the established method for determining canagliflozin concentration in plasma after oral administration.

4. CONCLUSION

The bioanalytical method was developed and validated to estimate the mean plasma concentration of drug after oral administration using internal standardization method. The optimized method was successfully applied to estimate the drug concentration in plasma after oral administration in rats. The better recovery of canagliflozin was attend. Due to higher sensitivity, accuracy, linearity and specificity, these HPLC methodology could thus be an appropriate and satisfactory for further determination of canagliflozin in plasma samples in the pharmacokinetic studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

The Institutional Animal Ethical Committee approved the experimental protocol.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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