

RP-HPLC Method Development and Validation for the Determination of Canagliflozin in Human Plasma

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Abstract : A simple, specific, sensitive, precise, selective and accurate reverse phase high performance liquid chromatographic method was developed for the determination of canagliflozin in human plasma as per US-FDA guidelines. Plasma samples were extracted by protein precipitation method using methanol as extracting solvent. The chromatographic separation was performed with WATERS EA874 (250 × 4.6 mm, 5 μm) column and mobile phase composed of 36.46 mM Acetate buffer: acetonitrile: methanol (30:50:20, v/v), pH 4.5 adjusted with acetic acid at a flow rate of 1.0 ml/min. Canagliflozin was detected at 290 nm with retention time of 5.1 min. Linearity was found to be 0.9929 over the range of 33.33 – 233.33 ng/ml and percentage recoveries were found to be 94.68 - 103.76 %. The validation was successfully performed by means of accuracy and precision, selectivity and specificity, linearity, recovery and stability under various conditions. This developed method can be successfully employed for the determination of Canagliflozin in human plasma.

Keywords: Canagliflozin, bioanalytical method, protein precipitation, RP-HPLC, US-FDA guidelines, stability studies.

Introduction:

Canagliflozin is white crystalline powder, almost odorless, slightly soluble in water, freely soluble in methanol and is chemically designated as (2S,3R,4R,5S,6R)-2-(3-{[5-(4-fluorophenyl)thiophen-2-yl]methyl}-4-methylphenyl)-6-(hydroxymethyl)oxane-3,4,5-triol having molecular formula C₂₄H₂₅FO₅S^[1, 2](Fig. 1).

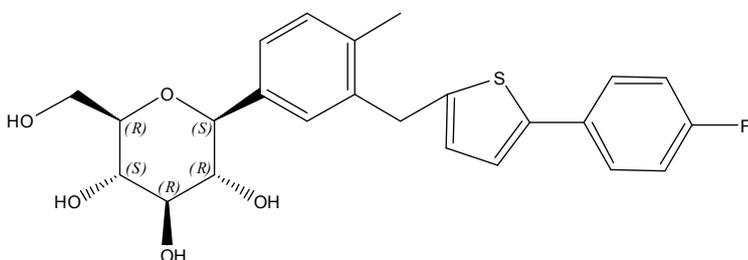


Fig. 1: Structure of Canagliflozin

Canagliflozin is official in USP^[3, 4]. It is the first compound which is a novel class of drugs that acts as Sodium glucose co-transporter 2 (SGLT-2) inhibitors. It inhibits renal glucose reabsorption by increasing urinary glucose excretion which leads to lowered renal threshold for glucose and reduced blood glucose levels in patients with type 2 diabetes mellitus^[2, 5-8]. Canagliflozin is an orally active, potent and exhibits approximately 200-

fold higher selectivity for SGLT-2 over SGLT-1^[9] and is approved at doses of 100 and 300 mg for the treatment of type 2 diabetes mellitus in many countries around the world wide^[2, 10].

The peak plasma concentration (C_{max}) obtained in 1-2 h because the oral absorption of canagliflozin is rapid. The steady state is achieved within 4-5 days after daily dose of administration. The absolute oral bioavailability of canagliflozin is 65 % and almost 99 % they bound to plasma protein mainly albumin. After single dose oral administration, the reported terminal half-life ranged from 10.6 - 13.1 h with a large volume of distribution that is 119 liter^[9, 11].

A detailed survey of literature revealed the determination of Canagliflozin by UV spectroscopic^[12-20], Spectrofluorometric^[21], HPLC with amperometry^[22], UHPLC-MS in biological fluids^[9] (i.e. human and rat plasma), Liquid Chromatography^[11, 23-30]. This study describes a new highly sensitive, rapid, simple, accurate, precise, reproducible, economical and stable visible chromatographic method for the determination of canagliflozin in human plasma.

Experimental:

Materials and Methods:

1. Chemicals and Reagents:

Canagliflozin pure drug was obtained from Xi'an Kingsmart Group Co. Limited (Xi'an City, China). HPLC grade methanol and acetonitrile were obtained from Merck India Ltd., Mumbai. Analytical grade acetic acid was obtained from Research-lab Fine Chem Industries, Mumbai and sodium acetate from Loba Chemi Pvt. Ltd., Mumbai. HPLC grade water produced by INFUSIL, India was used. Human plasma was obtained from Smt. Kashibai Navale Medical College and General Hospital, Narhe (Pune).

2. Preparation of stock solution:

Fresh standard stock solution (3000 µg/ml) was prepared by dissolving accurately weighed amount of pure material in methanol. Two separate stock solutions were prepared, one for calibration curve and one for Quality Control (QC) samples. All stock solutions were stored in refrigerator at 4°C.

➤ Calibration standards and QC sample preparation:

The working standards were prepared by diluting their respective amount of stock solution further by acetonitrile: methanol (50:50, v/v) for calibration curve and QC samples. The plasma calibration standards of canagliflozin were prepared at six different concentration levels by spiking 100 µl of their respective working standards in 200 µl of plasma to prepare final concentration levels ranged 33.33-233.33 ng/ml. Four different QC samples were also prepared by spiking 100 µl of their respective working standards in 200 µl of blank plasma as 33.33 ng/ml (LLOQ), 100 ng/ml (LQC), 166.66 ng/ml (MQC), 233.33 ng/ml (HQC). Both calibration standards and QC samples were stored in refrigerator at -80°C for validation study and sample analysis.

3. Instrumentation and Chromatographic conditions:

A quaternary HPLC system of 'Shimadzu' LC-2010 CHT model having UV detector, auto-sampler and sample cooler advance facility was used for the study. The chromatographic separation was performed with WATERS EA874 (250 × 4.6 mm, 5 µm) column. A double-beam UV-Visible spectrophotometer, model UV-1800 (Shimadzu, Japan) having two matched cells with 1 cm light path was used for detection of wavelength at maximum absorbance. A single pan electronic balance (Shimadzu, ATY 224) was used for weighing the samples. Calibrated volumetric glasswares (Borosil) were used in the study. Calibrated pH meters were used of Hanna instruments.

Mobile phase composed of 36.46 mM Acetate buffer: acetonitrile: methanol (30:50:20, v/v) and the flow rate was 1.0 ml/min. The pH (4.5) of buffer adjusted with acetic acid filtered through Millipore membrane filter (0.22 µm) and degassed within the ultrasonic bath (Spectra lab UCB 40) for 5 min. The injection volume

was 20 μ l and detection wavelength was selected 290 nm. The column oven temperature was 28°C and sample cooler temperature was 8°C.

4. Sample preparation:

All the plasma was thawed before sample preparation which was stored at -80°C. A simple protein precipitation method was employed for extraction of the analyte. An aliquot of 200 μ l of plasma with 100 μ l of working standards were added into clean tube and vortexed for 30 s followed by the addition of 1 ml of methanol into each tube. The vortex was repeated for the 1 min followed by centrifugation at 5000 rpm for 7 min at 4°C. After centrifugation the upper organic layer was transferred into clean glass tube and evaporated to dryness by gentle heating on water bath. The residue was reconstituted with 1 ml of mobile phase out of which 20 μ l was injected into the column for further analysis.

5. Method Validation ^[31]:

In accordance with international guidelines for bioanalytical method validation recommended by USFDA the assay validation was performed in human plasma in terms of selectivity and specificity, linearity and sensitivity, precision and accuracy, recovery and stability.

1. Selectivity and specificity:

Assay selectivity was evaluated in six blank plasma samples which were obtained from six different lots to test for any possible endogenous interference at the retention time of canagliflozin. Then the proposed extraction method was processed with these blank plasma samples. The response of co-eluting interference was evaluated by comparing their chromatograms with plasma spiked with the analyte at a LLOQ (33.33 ng/ml) level.

2. Linearity and sensitivity:

The assay linearity was determined by preparing calibration curve in human plasma, ranging from 33.33 to 2800 ng/ml concentration of the analyte. Calibration curves were obtained by plotting the peak area of analyte (*y*-axis) versus the concentration of analyte (*x*-axis) using weighted linear regression. The determination of coefficient of regression (R^2) was required to be ≥ 0.99 and the back calculated concentrations at each point have to be within $\pm 15\%$ deviation from the nominal value. The LLOQ was defined as the lowest concentration of the analyte in the plasma calibration curve that could be detected with an acceptable precision ($\leq 20\%$) and accuracy $\pm 20\%$.

3. Precision and accuracy:

Precision and accuracy was determined in human plasma at three different QC concentrations (LQC, MQC and HQC). The intraday precision and accuracy were determined by analyzing 3 replicates of each QCs on the same day, while the inter day precision and accuracy were determined by analyzing 9 replicates over three consecutive days. The deviation in the mean value of precision was limited to $< 20\%$ for the LLOQ and 15% for the other QC samples and accuracy was limited to be within $\pm 20\%$ for the lowest QC samples and $\pm 15\%$ for the other QC samples.

4. Recovery:

The extraction recovery of canagliflozin was determined at three QC levels (LQC, MQC and HQC) by comparing the peak area response of plasma spiked with the analyte prior to extraction with those spiked with the analyte after the extraction.

5. Stability:

The stability of canagliflozin was evaluated in human plasma by analyzing three replicates of two QC samples (LQC and HQC) under various storage conditions. All stability parameters were evaluated against freshly prepared plasma calibration curves. The freeze-thaw stability was determined after storing the spiked QC samples at 80°C and thawing at 25°C for three cycles. The bench-top stability of canagliflozin was

determined by processing and analyzing QC samples after keeping them for 8 h at room temperature. Long-term stability was determined by analyzing the spiked QC plasma samples which were stored at 80°C for 90 days. The stock solutions and working solutions of canagliflozin was also evaluated for their stability at room temperature for 6 h and 12 h at refrigerator temperature (4°C). The stability of processed samples, including the resident time in the autosampler was determined. The samples were considered stable if the deviation from the mean calculated concentration of quality control samples was found to be within the limits of accuracy ($\pm 15\%$) and precision ($\leq 15\%$).

Results and Discussion:

Optimization of chromatographic conditions:

Chromatographic separation by using different compositions of the mobile phase at different ratios and pH of the buffer was tried to achieve good resolution by optimizing the chromatographic parameters such as retention factor, selectivity (separation factor) and efficiency. The retention factor was optimized by adjusting the strength of organic modifiers in the mobile phase and the separation factor by optimizing the ratio of organic modifiers in the mobile phase, pH of the mobile phase and temperature of the column. Finally, the mobile phase consisting 36.46 mM Acetate buffer: acetonitrile: methanol (30:50:20, v/v) adjusted to pH 4.5 with acetic acid showed the best separation of the analyte was selected for analysis. The flow rate and ratio of buffer to organic phase were also optimized to achieve a maximum possible short runtime with an enhanced analytical signal response. The retention factor was comparatively low under the optimized chromatographic conditions, but high selectivity and efficiency resulted in separation of the analyte with high resolution. All the optimized chromatographic conditions are reported in Table 1.

Table 1: Optimized Chromatographic Conditions

Parameters	Specifications
HPLC Column	WATERS EA874 (250 mm \times 4.6 mm, 5 μ m)
Column temperature	28 C
Mobile Phase	Buffer: ACN: Methanol (30:50:20 v/v/v), pH 4.5
Acetate Buffer (pH 4.5)	Sodium acetate (2.99g) + Acetic acid (2N, 14.0 ml) make upto 1000 ml with distilled water
Diluent	Methanol
Elution mode	Isocratic elution
Flow rate	1.0 ml/min
UV detection	290 nm
Injection volume	20 μ L
Run time	6.25 min.
Retention time	5.1 min.

Optimization of sample preparation:

Sample extraction optimization is the most critical step to improve the sensitivity and recovery of analytes in biological fluids. With the time saving advantage and its simplicity, the protein precipitation method was tried initially using acetonitrile and methanol as protein precipitating agents and the result was satisfactory because of its low noise and interference by endogenous plasma at the retention time of the analyte. Finally methanol was selected as it produced a maximum recovery. The percentage extraction recovery of canagliflozin using methanol was found to be 99.88%.

Method Validation:

1. Selectivity and specificity:

There was no evidence of any endogenous interfering peak nearby the retention time of canagliflozin in all tested lots of blank plasma samples under the proposed chromatographic conditions. Representative chromatograms of blank plasma and plasma spiked with canagliflozin are shown in Fig. 2 and 3, respectively.

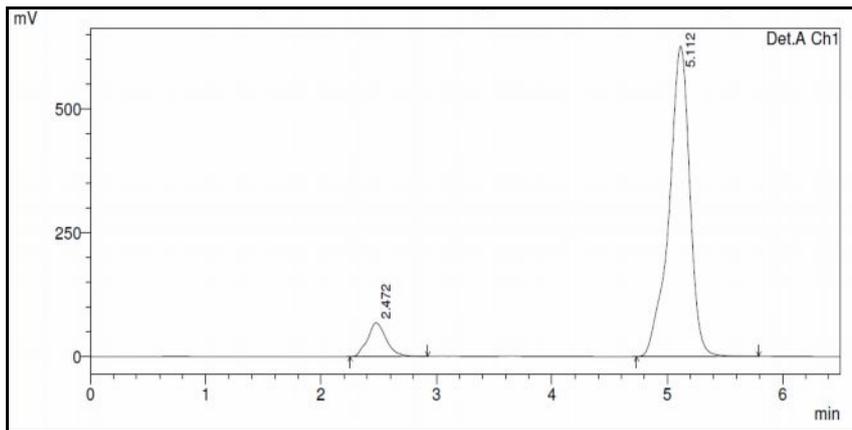


Fig. 2: Chromatogram of blank plasma

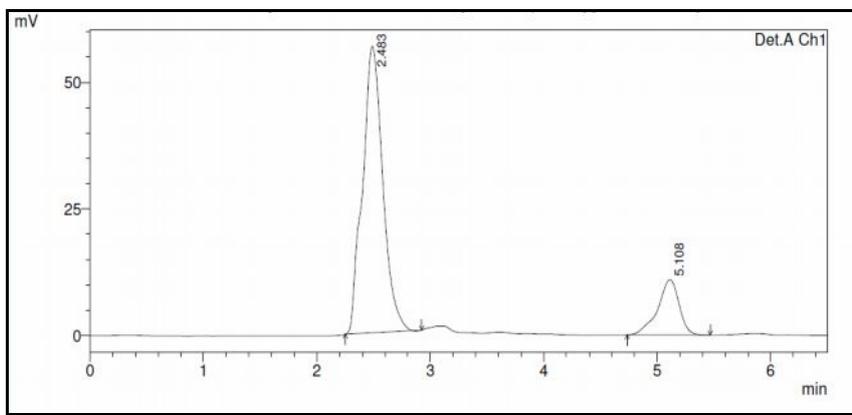


Fig. 3: Chromatogram of plasma spiked with Canagliflozin

2. Linearity and Lower limit of quantification (Sensitivity):

An excellent linear relationship was observed between peak areas of canagliflozin versus plasma concentrations over a range of 33.33-2800 ng/ml. The good linearity of plasma calibration curves was obtained with determination coefficient (R^2) 0.9929, slope (m) 29848 and y-intercept 112960. The back calculated concentrations at all points on the plasma calibration curve were within the $\pm 15\%$ of the nominal concentrations (Table 2). The assay offered an LLOQ of 33.33 ng/ml in human plasma. Canagliflozin was eluted at 5.1 min with a total run time of 6.25 min only. The linearity in plasma was achieved up to 2800 ng/ml (ULOQ) concentration.

Table 2: Linear Regression Data for Calibration Curves

Parameters	Specifications
Linearity range	33.33-233.33 ng/ml
R^2	0.9929
Slope	29848
Y-intercept	112960

3. Precision and accuracy:

The comparative results of intra- and inter-day precision and accuracy for canagliflozin at three QC levels (LQC, MQC and HQC) by RP-HPLC method in human plasma is presented in Table 3. In the present RP-HPLC assay, the intra-day and inter-day precision values (expressed as % CV) were $\leq 0.08\%$ and $\leq 0.32\%$, respectively. Similarly, the intra-day and inter-day accuracy ranged from 95.04-103.06 % and 97.81-110.61 %, respectively. The results showed that the assay met the desired level of acceptance criteria similar to previously

reported UHPLC-MS/MS assay and hence was considered accurate and precise for application in determination of canagliflozin in human plasma.

Table 3: Precision and Accuracy

Parameters	Accuracy			Precision					
	LQC	MQC	HQC	Intraday			Inter-day		
				LQC	MQC	HQC	LQC	MQC	HQC
SD	43090.53	14354.06	52861.44	2477.56	93.63	5071.89	11985.51	9830.54	10398.59
% CV	1.47	0.31	0.74	0.08	0.002	0.072	0.32	0.18	0.13

4. Recovery:

The %mean recovery of canagliflozin using the proposed extraction method at LQC, MQC and HQC levels were found to be 101.18 %, 94.69 % and 103.77 % with % CV of 0.09 %, 0.58 % and 0.95 %, respectively. This result indicates that the recovery of canagliflozin by the protein precipitation method using methanol as extracting solvents was in the acceptable range, consistent and concentration independent. So the extraction process of the plasma sample was sufficient to isolate the canagliflozin from human plasma volume.

5. Stability:

The comparative stability results of canagliflozin under different storage conditions (freeze-thaw, autosampler, post-preparative, short-term and long-term) by RP-HPLC assay in human plasma is summarized in Table 4. The results demonstrated that canagliflozin spiked plasma was stable during three cycles of freeze-thaw, 48 h in an autosampler after post-preparation, at least 8 h at room temperature, and up to 90 days at around 80°C. The stock solutions and working standard of canagliflozin were also found to be stable for 15 days at refrigerator temperature (below 10°C). The deviation of meantest responses (% CV) was within $\pm 15\%$ at LQC and HQC concentration levels. Thus, there is no indication of canagliflozin instability in human plasma samples which can affect the assay performance.

Table 4: Stability Studies

Parameters	Specifications	Concentration levels	SD	% CV	% Accuracy
Freeze-thawed	72 h	LQC	72183.32	1.99	104.89
		HQC	9310.74	0.12	114.92
Short term or Bench-top	6 h	LQC	47690.11	1.62	102.65
		HQC	13366.93	0.19	104.05
	12 h	LQC	4229.08	0.11	108.20
		HQC	7783.26	0.1	114.26
	24 h	LQC	6703.31	0.18	108.09
		HQC	4676.74	0.06	105.25
Long term	First day	LQC	1208.19	0.04	101.32
		HQC	23989.54	0.34	103.54
	Last day (72 h)	LQC	34161.26	0.92	108.24
		HQC	11222.28	0.14	114.83
Standard stock solution	2 h	LQC	22018.37	0.07	104.96
		HQC	113987.85	0.16	102.91
	6 h	LQC	41379.69	0.13	104.66
		HQC	182955.59	0.25	102.89
Post-operative	24 h	LQC	11985.51	0.32	107.81
		HQC	10398.59	0.13	105.72

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