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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF CANAGLIFLOZIN IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: A validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for canagliflozin in human plasma along with stability studies.

Methods: The chromatographic separation of canagliflozin was performed on Zorbax XDB phenyl (75 × 4.6 mm, 3.5 mm) using methanol:acetate buffer (80:20 v/v) at a flow rate of 1.0 ml/min. The LC–MS/MS system consists of API 4000 triple quadrupole mass spectrometer equipped with turbospray ionization and an AS8020 automatic sample injector.

Results: The retention time of canagliflozin was 1.15 min and total runtime was 2 min. The multiple reaction monitoring was 462.5/267.1 (m/z) for canagliflozin and 466.4/267.2 (m/z) for internal standard (canagliflozin D_4), respectively. The method was linear over the range of 10–7505 ng/ml. The calculated slope ranged from 0.0451 to 0.0502 and intercepts from 0.0102 to 0.0456 with coefficients of the determination of 0.9970. The overall mean recovery of internal standard and canagliflozin was 76.66 and 79.77, respectively.

Conclusion: The method was successfully validated and it was found to be within the limits for accuracy, precision, and linearity and it is stable under analytical conditions used.

Keywords: Canagliflozin, Liquid chromatography–tandem mass spectrometry, Human plasma, Liquid–liquid extraction, Validation, Stability studies.

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INTRODUCTION

Canagliflozin chemically is (2S.3R, 4R, 5S, 6R)-2-(3-{[5-(4-flurophenyl) thiophenyl) thiopen-2-yl] methyl}-4 methyl phenyl)-6-(hydroxy methyl) oxane-3, 4, 5,-triol represented in Fig. 1 (Drug bank) [1]. The molecular formula is $C_{24}H_{25}FO_5S$ and molecular weight is 444.52 g/mol. Canagliflozin is classified as SGLT-2 inhibitor, a new class of antidiabetic drug having an insulin-dependent mechanism that offers a considerable advantage of increasing urinary glucose excretion without inducing hypoglycemia [2]. Several analytical methods such as ultraviolet [3], high-performance liquid chromatography (HPLC) [4-7], high-performance thin-layer chromatography [8], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [9-11] have been developed for analysis of canagliflozin. There are methods developed for canagliflozin in rat plasma. However, there is no method reported for canagliflozin in human plasma along with stability studies. This study describes that a validated LC–MS/MS method was developed for canagliflozin in human plasma along with stability studies.

METHODS

Chemicals and reagents

Canagliflozin and internal standard (canagliflozin D_4) were obtained from Piramal Healthcare. K3EDTA plasma was from local suppliers, acetonitrile and methanol were of HPLC grade, ammonium acetate (GR grade) was used, and water was from Milli Q system.

Instrumentation

The HPLC separation was achieved on Zorbax XDB phenyl (75×4.6 mm, 3.5 mm) using methanol:acetate buffer (80:20 v/v) at a flow rate of 1.0 ml/min. The injection volume was 10 µl and the column temperature was 30°C. The samples were held at 5±3°C in an autosampler. The runtime was 2.0 min. The LC–MS/MS system consists of API 4000 triple quadrupole mass spectrometer equipped with turbospray ionization and an AS8020 automatic sample injector. The multiple reaction monitoring (MRM) was 462.5/267.1 (m/z) for canagliflozin and 466.4/267.2 (m/z) for internal standard (canagliflozin D_4), respectively. The temperature of the capillary was 50°C and the dwell time was 100 millisecond or ms.

Preparation of standards and quality control (QC) samples

Stock solution of canagliflozin was prepared in methanol to get concentration of 5 µg/ml. The calibration curve standard solution was prepared by further diluting the stock solution in methanol to the following analytical condition (10, 25, 150, 375, 750, 1875, 3750, 6000, and 7500 ng/ml) for canagliflozin. The internal standard working solution was prepared by diluting stock solution in methanol to 5000 ng/ml. QC samples were prepared in the same manner from the QC stock to get final concentration of 28 (LQC), 706 middle QC (MQC), and 5700 high QC (HQC) in plasma. QC samples were stored in deep freezer with study samples and include with all validation and sample analysis runs.

Extraction procedure

To a glass tube containing 300 µl of plasma sample, added 50 µl of 2000 ng/ml internal standard working solution. The sample was mixed on a vortex mixer for approximately 5 s. Then, 2.0 µl of tertiary butyl methyl ether was added to the vials and extracted for a period of 15 min or rotospin at 40 rpm. The vials were centrifuged at 4500 rpm at 4±1°C for 5 min. Finally, the samples (1.8 µl) were eluted into a deep well collection plate evaporated to dryness under nitrogen at 40±5°C and reconstituted in 300 µl of solution of mixture of acetonitrile:phosphate buffer (80:20%) vortexed for about 10 s, and finally, 10 µl of each reconstituted sample extract was injected into LC–MS/MS.

Assay validation

The method was validated as per Food and Drug Administration guidance for bioanalytical method validation [12].

Accuracy and precision

The accuracy and precision of the proposed method were determined using QC samples (low, medium, and high) over the concentration of 28–5750 ng/ml for assay precision and accuracy. Six QC validation levels such as DQC, LQC, MQC3, MQC2, MQC1, and HQC were tested. The accuracy and intraday precision of the assay method were performed on three different runs, each run containing duplicate full calibration curves and six samples for each of the six QC levels. The recovery from human plasma during extraction was determined at LQC, MQC1, and HQC levels for canagliflozin by comparing the response ratios in human plasma sample with those of QC sample spiked in the supernatant of the extracted blank plasma. The LLOQ was assessed using plasma samples at 10 ng/ml for canagliflozin, the lowest concentration in the standard curves. Six different lots of control human plasma were spiked to obtain the six LLOQ samples. The LLOQ samples were processed and analyzed with standard curves and QC samples.

The matrix effect was determined at low- and high-level QC for canagliflozin. The absolute matrix factors for three QC samples were determined by comparing the peak area of the QC sample spiked in the mobile phase with those in the supernatant of extracted blank plasma.

Stability studies

The stability studies of canagliflozin in human plasma were evaluated using QC samples (low, medium, and high concentration) under various conditions. The autosampler stability was evaluated by analyzing QC samples that had been stored under conditions (5±3°C) and room temperature for 3 days. The long-term stability was also evaluated by analyzing QC samples that had been stored at 2–8°C for 7 days. Freeze-thaw stability was also evaluated by analyzing HQC and LQC samples after freezing at −28±5°C and thawing at room temperature 5 times.

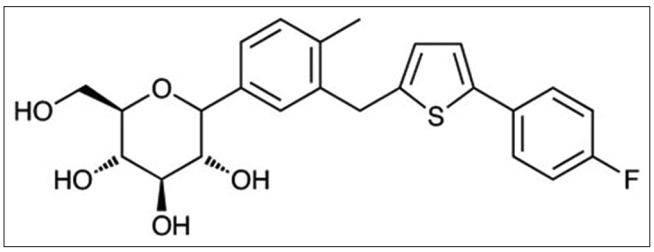

Fig. 1: Chemical structure of canagliflozin

The stability was established if the average of the six determinations was within 15% and no obvious trend was observed.

RESULTS AND DISCUSSION

In the present study, LC–MS/MS assay was developed for positive ionization which was evaluated. The full scan mass spectrum of canagliflozin and internal standard in the positive MRM is presented in Figs. 2 and 3. The reliability of the method was assessed on the basis of linearity, precision, selectivity, accuracy, recovery, and carryover test. Finally, the chromatographic separation was carried out on a combination of methanol:acetate buffer (80:20 v/v) at a flow rate of 1.0 ml/min which resulted in a separation time of 1.15 min for analyte and internal standard.

Accuracy and precision

The interbatch coefficient of variation ranged from 2.86 to 5.61 and percentage accuracy ranged from 101.61 to 109.86 for canagliflozin. The results for within and between in batch precision for LQC, MQC, and HQC should be <15.00%, and for the LLOQ, it should be <20.00%. The intrabatch coefficient of variation ranged from 2.80 to 4.97 and the percentage accuracy ranged from 102.04 to 110.38% for canagliflozin. The precision ranged from 2.58 to 3.39%. The results prove that the canagliflozin and internal standard can remain in autosampler for 67 h 15 min, without showing a significant loss indicates that the sample should be analyzed within this period. The results are shown in Table 1.

Linearity

The method was linear over the range of 10–7505 ng/ml. The calculated slope ranged from 0.0451 to 0.0502 and intercepts from 0.0102 to 0.0456 with coefficients of the determination of 0.9970 or higher.

Recovery

The mean recovery of canagliflozin and canagliflozin D_4 (internal standard) was evaluated by comparing peak mean peak response of LQC, MQC1, and HQC sample to those of diluted aqueous solution. The overall mean recovery of internal standard and canagliflozin was 76.66 and 79.77, respectively. The overall percentage coefficient of variation was 3.94. This indicates that the method has good recovery of both analyte and internal standard. The results are shown in Tables 2 and 3.

Matrix effect

No significant matrix effect was observed in all the eight batched for canagliflozin at LQC and HQC concentrations. The precision for internal standard normalized matrix factor at LQC and HQC level was found to be 2.46% and 3.84%, respectively. The precision of internal standard normalized matrix at each level (HQC and LQC) should be <15.00. The above-reported method showed that no matrix effect was found for plasma and shown in Table 4.

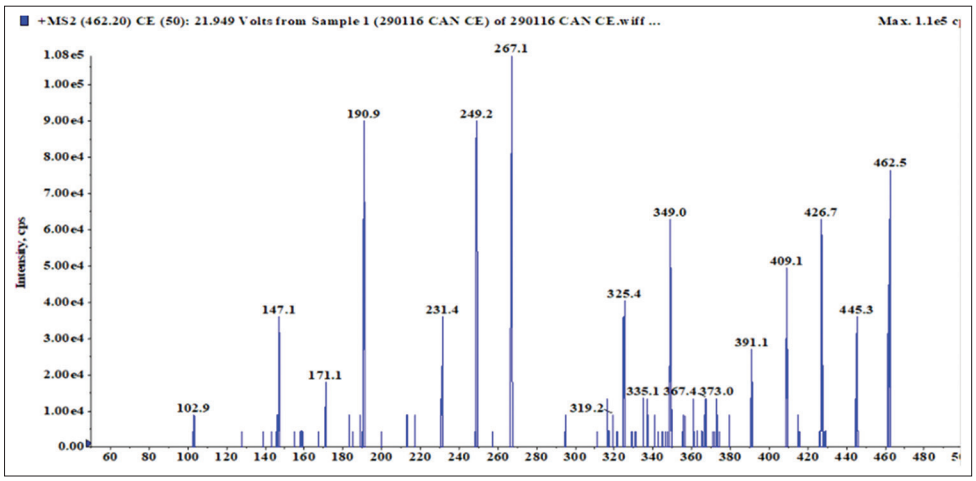

Fig. 2: Mass spectra of canagliflozin

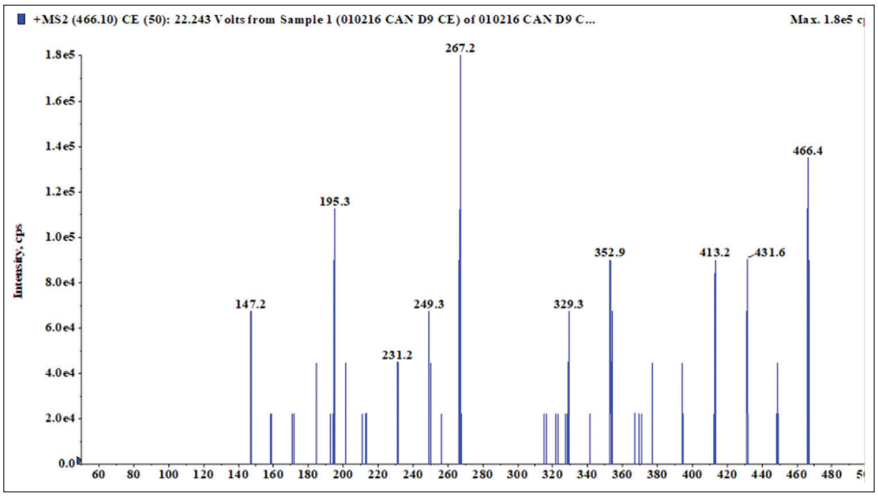

Fig. 3: Mass spectra of canagliflozin D_4

Table 1: Accuracy and precision of canagliflozin

S. No.	Q.C nom. conc. (ng/ml)	Mean (ng/ml)		Precision (CV %)		Accuracy (%)		SD	
		Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
1.	LQC (28.89)	29.48	30.25	4.89	4.15	102.0	104.73	1.44	1.25
2.	MQC (705.22)	778.4	774.75	3.11	2.86	110.3	109.86	24.22	22.15
3.	HQC (5750.45)	6007.72	5843.0	2.80	4.42	104.4	101.61	168.3	258.4

Q.C nom. conc.: Quality control nominal concentration. CV: Coefficient of variation, SD: Standard deviation, HQC: High-quality control, MQC: Middle-quality control, LQC: Low-quality control, QC: Quality control

Table 2: Recovery for canagliflozin

S. No.	HQC		MQC1		LQC	
	Post-extracted response	Extracted response	Post-extracted response	Extracted response	Post-extracted response	Extracted response
1.	4,002,365	3,023,654	2,869,574	2,236,577	31,564	25,645
2.	3,485,623	3,125,678	2,798,654	2,045,689	30,214	24,587
3.	3,698,756	3,256,891	2,856,457	2,145,689	32,564	21,457
4.	3,789,562	3,369,871	2,903,654	2,365,894	33,456	25,487
5.	3,895,647	3,045,689	2,778,965	2,265,436	31,265	26,354
6.	3,957,863	3,256,489	2,812,654	2,154,879	30,125	25,487
Mean	3,804,969.3	3,179,712.0	2,836,659.7	2,202,360.7	31,531.3	24,836.2
SD	191,866.99	136,563.31	47,566.60	111,269.59	1308.12	1748.49
% CV	5.04	4.29	1.68	5.05	4.15	7.04
% mean recovery		79.99				
SD		3.149				
% CV		3.94				

HQC: High-quality control, MQC: Middle-quality control and LQC: Low-quality control, CV: Coefficient of variation, SD: Standard deviation

Table 3: Recovery for internal standard

S. No.	HQC		MQC1		LQC	
	Post-extracted response	Extracted response	Post-extracted response	Extracted response	Post-extracted response	Extracted response
1.	155,645	112,356	165,234	125,645	225,687	190,365
2.	145,687	110,234	160,324	120,364	245,897	185,641
3.	149,654	120,236	166,354	130,324	201,365	189,654
4.	150,234	115,234	160,324	131,256	223,654	175,654
5.	151,234	100,364	159,654	129,365	236,545	177,563
6.	152,364	108,654	185,364	125,847	232,254	170,235
Mean	150,803.0	111,179.7	166,209.0	127,133.5	227,567.0	181,518.7
SD	3282.83	6694.63	9799.19	4046.00	15,125.82	8231.62
% CV	2.18	6.02	5.90	3.18	6.65	4.53
% mean recovery		76.66				
SD		3.019				
% CV		3.94				

HQC: High-quality control, MQC: Middle-quality control, LQC: Low-quality control, SD: Standard deviation, CV: Coefficient of variation

Sensitivity

The lowest limit of reliable quantification of canagliflozin in human plasma set at the concentration of the LLOQ is 10.13 ng/ml. The precision and accuracy for canagliflozin at this concentration was found to be 2.83% and 95.65%.

Stability

The stability of canagliflozin and internal standard was evaluated in plasma under different conditions such as freeze-thaw stability, bench-top stability, autosampler stability, and long-term stability. All the stabilities were carried out at two concentrations (28.893 ng/ml and 5750.456 ng/ml) as low and high concentration values with six determinations for each stability test along with calibration curve standards.

Table 4: ISTD normalized matrix factor

S. No.	HQC	LQC
1.	1.05	1.05
2.	1.11	1.00
3.	1.12	1.04
4.	1.09	1.01
5.	1.05	0.97
6.	1.16	1.03
7.	1.11	1.01
8.	1.04	1.02
Mean	1.091	1.016
SD	0.0419	0.0250
% CV	3.84	2.46

ISTD: Internal Standard, SD: Standard deviation, HQC: High-quality control, LQC: Low-quality control, CV: Coefficient of variation

The refrigerated stock solution stability of canagliflozin was carried out by injecting six replicates of internal standard. The precision ranged from 2.04% to 2.94% and percentage of stability was found to be 99.29%. The internal standard precision ranged from 0.95% to 1.65% and percentage of stability was found to be 98.49%.

The autosampler stability of canagliflozin was performed by injecting six sets of QC samples (LQC and HQC) and placed in autosampler for 67 h 15 min. The percentage stability was 105.33% and 107.07% for HQC and LQC; percentage mean accuracy was 105.54% and 106.33%, respectively. The results are shown in Table 5.

The analytes were found to be stable in dry as well as wet extract. The dry extract stability was carried out at room temperature, whereas wet extracted solubility was carried out at refrigerator temperature (2–8°C). The wet extract stability for refrigerator temperature has been proved at 57 h 45 min, ranged from 105.51% to 106.67% and precision ranged from 5.51% to 7.67%, respectively. The values are shown in Table 6.

The dry extract solubility has been proven at room temperature for 10 h 15 min, ranged from 100.41 to 104.925 and precision ranged from 1.98 to 7.27%, respectively. The values are shown in Table 7.

The freeze-thaw stability of canagliflozin was carried out for five cycles at −28±5°C. The percentage mean stability was 92.04–101.58% and precision was 3.48–4.57%, respectively. The results are shown in Table 8.

The bench-top stability was carried out using six sets, each of LQC and HQC was determined at 17 h 5 min. The percentage mean accuracy was

Table 5: Autosampler stability

S. No.	Back calculated concentration (ng/ml)			
	Comparison samples (FQC)	Stability samples	Comparison samples (FQC)	Stability samples
1.	5525.235	5856.325	27.258	30.258
2.	5986.324	5936.235	28.698	31.254
3.	5635.231	6025.321	28.987	29.654
4.	6023.254	6125.364	29.654	31.257
5.	5864.365	6235.241	30.258	32.254
6.	5789.654	6234.214	26.365	29.654
Mean	5804.0105	6068.7833	28.5367	30.7218
SD	195.63077	156.76314	1.46899	1.04046
% CV	3.37	2.58	5.15	3.39
% mean accuracy	100.19	105.54	99.32	106.33
% mean stability	105.33		107.06	
% bias	5.33		7.06	

SD: Standard deviation, CV: Coefficient of variation, FQC: Final quality control

Table 6: Wet extract stability at refrigerator temperature

S. No.	Back calculated concentration (ng/ml)			
	Comparison samples	Stability samples	Comparison samples	Stability samples
1.	5525.235	5865.234	27.258	30.254
2.	5986.324	5965.321	28.698	31.254
3.	5635.231	6025.321	28.987	29.365
4.	6023.254	6125.324	29.654	30.269
5.	5864.365	6235.214	30.258	32.254
6.	5789.654	6258.547	26.365	31.987
Mean	5804.0105	6079.1602	28.5367	30.8972
SD	195.63077	155.10380	1.46899	1.12344
% CV	3.37	2.55	5.15	3.64
% mean accuracy	100.19	105.72	99.32	106.94
% mean stability	105.51		107.67	
% bias	5.51		7.67	

% mean accuracy: Percentage mean accuracy, % CV: % Coefficient of variation, SD: Standard deviation, FQC: Final quality control

Table 7: Dry extract stability at room temperature

S. No.	Back calculated concentration (ng/ml)			
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
1.	5525.235	6023.542	27.258	25.365
2.	5986.324	5963.254	28.698	28.657
3.	5635.231	6025.321	28.987	29.657
4.	6023.254	5898.365	29.654	31.254
5.	5864.365	6123.254	30.258	27.687
6.	5789.654	6235.247	26.365	30.257
Mean	5804.0105	6044.8305	28.5367	28.8128
SD	195.63077	119.45658	1.46899	2.09461
% CV	3.37	1.98	5.15	7.27
% mean accuracy	100.19	105.12	99.32	99.72
% mean stability	104.92		100.41	
% bias	4.92		0.41	

SD: Standard deviation, CV: Coefficient of variation, SD: Standard deviation, FQC: Final quality control

Table 8: Freeze-thaw stability

S. No.	Back calculated concentration (ng/ml)			
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
1.	5525.235	5026.354	27.258	28.654
2.	5986.324	5124.258	28.698	29.354
3.	5635.231	5264.258	28.987	27.354
4.	6023.254	5525.365	29.654	29.657
5.	5864.365	5654.258	30.258	30.258
6.	5789.654	5458.254	26.365	28.654
Mean	5804.0105	5342.1245	28.5367	28.9885
SD	195.63077	244.02069	1.46899	1.00871
% CV	3.37	4.57	5.15	3.48
% mean accuracy	100.93	92.90	98.77	100.33
% mean stability	92.04		101.58	
% bias	−7.96		1.58	

% mean stability: Percentage mean stability, % mean accuracy: Percentage mean accuracy. SD: Standard deviation, CV: Coefficient of variation, FQC: Final quality control

Table 9: Long-term stability

S. No.	Back calculated concentration (ng/ml)			
	Comparison samples (FQC)	**Stability samples**	**Comparison samples (FQC)**	**Stability samples**
1.	5636.354	6235.354	28.365	29.365
2.	5863.254	6125.324	29.365	30.321
3.	5963.214	6023.254	30.254	31.254
4.	6123.547	5965.321	31.254	32.254
5.	6025.321	6032.214	29.365	29.367
6.	6124.254	6125.234	31.254	30.254
Mean	5955.9907	6084.4502	29.9762	30.4692
SD	185.48004	96.71743	1.15636	1.12284
% CV	3.11	1.59	3.86	3.69
% mean accuracy	103.04	105.81	104.25	105.46
% mean stability	102.69		101.16	
% bias	2.69		1.16	

SD: Standard deviation, CV: Coefficient of variation, FQC: Final quality control

108.82–109.21%, respectively, and the precision range was from 2.04 to 2.47%, respectively.

The long-term matrix stability of QC samples was stored at −28±5°C for 91 days which was assessed. The percentage stability was found to be 101.16–102.69%; coefficient of variation was 1.59–3.69. These values indicate that the canagliflozin is stable for at least 91 days; it should be analyzed within this period. The results are shown in Table 9.

CONCLUSION

The results of matrix effect, linearity, precision, accuracy, stabilities, and recovery were in the acceptable range as per guidance for industry-bioanalytical method validation. The LC–MS/MS method described above is valid for the estimation of canagliflozin in human plasma over a range of 462.500/267.100 with the detection of canagliflozin (m/z) and internal standard canagliflozin D_4 466.400/267.200 (m/z) in positive ion mode.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally to the paper.

CONFLICTS OF INTEREST

The author declares that they have no conflicts of interest.

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