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 D4), 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.

INTRODUCTION

Canagliflozin chemically is [2S,3R,4R,5S,6R]−2−{3−{5−(4-flurophenyl) thiophenyl}thioen−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 C21H18FO4S and molecular weight is 444.52 g/mol. Canagliflozin is classified as SGTL-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 D4) 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 D4), respectively. The temperature of the capillary was 50°C and the dwell time was 100 milliseconds 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 LQC and MQC samples after freezing at −28±5°C and thawing at room temperature 5 times.

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 D₄ (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. 1: Chemical structure of canagliflozin

Fig. 2: Mass spectra of canagliflozin
Table 1: Accuracy and precision of canagliflozin

| S. No. | QC nom. conc. (ng/ml) | Mean (ng/ml) | Precision (CV %) | Accuracy (%) | SD |
|--------|-----------------------|--------------|------------------|--------------|----|
|        | Intra | Inter | 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 | 3,369,871 | 2,856,457 | 2,145,689 | 32,564 | 21,457 |
| 4.     | 3,789,562 | 3,045,689 | 2,903,654 | 2,365,894 | 30,214 | 24,587 |
| 5.     | 3,895,647 | 3,256,489 | 2,778,965 | 2,265,436 | 31,626 | 26,354 |
| 6.     | 3,957,863 | 3,179,712 | 2,812,654 | 2,154,879 | 31,513 | 24,856.2 |
| Mean   | 3,804,969.3 | 3,179,712 | 2,836,659.7 | 2,202,360.7 | 31,531.3 | 24,856.2 |
| SD     | 191,866.99 | 136,563.31 | 47,566.60 | 111,269.59 | 31,531.3 | 24,856.2 |
| % CV   | 5.04 | 1.68 | 3.149 | 4.29 | 3.94 |
| % mean recovery | 79.99 | 79.99 | 79.99 |

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     | 152,828.3 | 669,643 | 979,19 | 494,00 | 15,125.82 | 823,162 |
| % CV   | 2.18 | 6.02 | 5.90 | 3.18 | 6.65 | 4.53 |
| % mean recovery | 76.66 | 76.66 | 76.66 |
| SD     | 3.019 | 3.019 | 3.019 |
| % CV   | 3.94 | 3.94 | 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.

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 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 in 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 freezethaw 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.

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The autsamplener 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 in 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 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

| S. No. | Back calculated concentration (ng/ml) | Stability samples | Comparison samples (FQC) | Stability samples |
|-------|--------------------------------------|-------------------|--------------------------|-------------------|
| 1.    | 5525.235                             | 27.258            | 30.258                   |
| 2.    | 5986.324                             | 28.698            | 31.254                   |
| 3.    | 5635.231                             | 28.987            | 29.654                   |
| 4.    | 6023.254                             | 30.258            | 32.254                   |
| 5.    | 5864.365                             | 26.365            | 29.654                   |
| Mean  | 5804.0105                            | 28.5367           | 30.7218                  |
| SD    | 3.37                                 | 1.46899           | 1.04046                  |
| %CV   | 100.19                               | 5.15              | 3.39                     |
| % mean accuracy | 105.54 | 99.32 | 106.33 |
| % mean stability | 105.33 | 107.06 |
| % bias | 5.33 | 7.06 |

| S. No. | Back calculated concentration (ng/ml) | Stability samples | Comparison samples (FQC) | Stability samples |
|-------|--------------------------------------|-------------------|--------------------------|-------------------|
| 1.    | 5525.235                             | 27.258            | 30.258                   |
| 2.    | 5986.324                             | 28.698            | 31.254                   |
| 3.    | 5635.231                             | 28.987            | 29.654                   |
| 4.    | 6023.254                             | 30.258            | 32.254                   |
| 5.    | 5864.365                             | 26.365            | 29.654                   |
| Mean  | 5804.0105                            | 28.5367           | 30.7218                  |
| SD    | 3.37                                 | 1.46899           | 1.04046                  |
| %CV   | 100.19                               | 5.15              | 3.39                     |
| % mean accuracy | 105.54 | 99.32 | 106.33 |
| % mean stability | 105.33 | 107.06 |
| % bias | 5.33 | 7.06 |

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.

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 in 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 stability 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 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 D₄ 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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