A Novel and Rapid RP-HPLC Quantitative Method for the estimation of Canagliftin in Human Plasma

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ABSTRACT
A simple, precise and accurate method was developed for the quantitative estimation of Canagliftin in human plasma using Dapagliftin as internal standard by Reverse Phase-High Performance Liquid Chromatographic technique. Chromatographic conditions were of stationary phase Phenomenex Luna C18 (2) (150 x 4.6 mm, 5m), Mobile phase 0.01 N Potassium dihydrogen Phosphate buffer pH (3.5±0.05) : acetonitrile in the ratio of 45:55 and flow rate at 1.0 ml/min, detection wave length was UV 222 nm, column oven temperature was maintained at 30°C, and sample injection volume of 10 μL. Retention time of Canagliftin was found to be about 8.7 min. Coefficient of Variation for Canagliftin peak was 3.15 %, % recovery was 94.58 %. The linearity of method was studied from 0.06 μg/ml to 2.4 μg/ml (R² = 0.999). The Signal to Noise ratio of lower limit of quantification (0.06 μg/ml) was found to be 50. The proposed bio-analytical method was validated by following ICH guidelines.

INTRODUCTION
Diabetes mellitus affects 425 million people in the world, which accounts for this number rising to around 629 million diabetes globally by 2045 (Effien, 2019). Sodium-Glucose co-transporter 2 (SGLT2) inhibitors are a novel approach in the treatment plan of type 2 diabetes mellitus (Devineni and Polidori, 2015). Canagliftin is the first SGLT2 inhibitor available on the market (2015) which reduces systolic blood pressure and has a low risk of hypoglycemia (Inzucchi et al., 2012). Canagliftin hemihydrate (Figure 1) is chemically 1-(beta-d-glucopyranosyl)-4-methyl-3-[5-(4-fluorophenyl)-2-thienylmethyl]benzene hemihydrates with a molecular weight of 453.52 g/mol. For the quantification of Canagliftin in pharmaceutical dosage forms and biological fluids only few analytical methods like LC-MS/MS (Saibaba et al., 2018), UV (Pooja et al., 2018), RP-HPLC (Sen et al., 2018; Ladva et al., 2016; Krishna et al., 2018; D’souza et al., 2016; Sreenivasulu et al., 2014; Marella et al., 2017), HPTLC method (Kaur et al., 2016; Kaur and Wakode, 2017) are reported. Simple, rapid (to reduce analytical down time in turn revenue) and
cost effective bio-analytical method development is a thirst area of importance in the Pharmaceutical Industry. Hence a simple, precise, accurate, sensitive, selective and robust RP-HPLC bioanalytical method was developed and validated as per ICH guideline for the estimation of Canagliftslozin, in human plasma is reported in this article.

**Figure 1: Structural Formula of Canagliftslozin hemihydrate**

**MATERIALS AND METHODS**

**Reagents and Chemicals** Canagliftslozin hemihydrate and Dapagliftslozin were obtained as gift samples. HPLC grade Acetonitrile and water were purchased from the local vendor (Make: Merck, Mumbai, India). Potassium di-hydrogen phosphate (ACS grade) and ortho-phosphoric acid (ACS Grade) were procured from the local supplier (Make: Rankem, Hyderabad, India).

**Instrumentation and Chromatographic Condition**

Chromatographic analysis was performed on Waters Alliance HPLC 2695 model equipped with quaternary pumps, Photodiode array detector and auto sampler. Empower 2 Software is used thorough out the analysis. The HPLC column used was Phenomenex Luna C18 (2) (4.6 x 150 mm, 5m). The mobile phase consist of 0.01N potassium di-hydrogen phosphate in water (pH 3.5 ± 0.04 adjusted with 10% ortho-phosphoric acid solution) and Acetonitrile in the ratio of (45:55) % v/v. Filtered the mobile phase through 0.45 μ membrane filter under vacuum and degassed. Mobile phase flow rate was set at 1.0 ml/min and column oven temperature was set at 30°±2°C. The detector wavelength was set at UV 222 nm. The auto-sample cooler temperature was set at 5°±2°C. Chromatographic run time was monitored for 15 min.

**Preparation of Solutions**

**Preparation of diluent** Mixture of 0.01N potassium di-hydrogen phosphate in water (pH 3.5 ± 0.1
Table 1: Evaluation of System Suitability

| S No | Analyte (Canagliflozin) Area | Retention Time of Canagliflozin (min) | Internal standard (Dapagliflozin) Area | Retention Time for Internal standard (Dapagliflozin) (min) | Area Ratio (Analyte / Internal Standard) |
|------|-----------------------------|---------------------------------------|----------------------------------------|----------------------------------------------------------|------------------------------------------|
| 1    | 135280                      | 8.75                                  | 1073287                                | 4.15                                                     | 0.1260                                   |
| 2    | 136747                      | 8.75                                  | 1066933                                | 4.15                                                     | 0.1282                                   |
| 3    | 131298                      | 8.77                                  | 1063306                                | 4.15                                                     | 0.1235                                   |
| 4    | 134214                      | 8.77                                  | 1046126                                | 4.15                                                     | 0.1283                                   |
| 5    | 137520                      | 8.78                                  | 1061621                                | 4.16                                                     | 0.1295                                   |
| 6    | 134681                      | 8.78                                  | 1050177                                | 4.16                                                     | 0.1282                                   |
| Mean Area | 8.767                      | NA                                    | NA                                     | 4.153                                                    | 0.1273                                   |
| SD   | 0.0173                      |                                       |                                       | 0.0055                                                   | 0.00218                                   |
| %CV  | 0.20                        |                                       |                                       | 0.13                                                     | 1.71                                      |

Table 2: Data for Calibration Curve of Canagliflozin

| S No. | Standard Solution 1 | Standard Solution 2 | Standard Solution 3 | Standard Solution 4 | Standard Solution 5 | Standard Solution 6 | Standard Solution 7 | Standard Solution 8 |
|-------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|       | Concentration Found (µg/ml) |                      |                      |                      |                      |                      |                      |                      |
| 1     | 0.057               | 0.118               | 0.177               | 0.476               | 1.181               | 1.428               | 1.918               | 2.386               |
| 2     | 0.063               | 0.111               | 0.182               | 0.480               | 1.194               | 1.432               | 1.911               | 2.399               |
| 3     | 0.059               | 0.121               | 0.169               | 0.486               | 1.203               | 1.446               | 1.921               | 2.462               |
| Mean  | 0.0596              | 0.1166              | 0.1760              | 0.4806              | 1.192               | 1.4353              | 1.9166              | 2.415               |
| SD    | 0.0031              | 0.0052              | 0.0066              | 0.0051              | 0.0111              | 0.0095              | 0.0052              | 0.0406              |
| %CV   | 5.12                | 4.40                | 3.73                | 1.05                | 0.93                | 0.66                | 0.27                | 1.68                |
| Mean Accuracy (%) | 99.44              | 97.22               | 97.78               | 100.14              | 99.39               | 99.68               | 99.83               | 100.65              |

Table 3: Within run Accuracy and Precision data for Canagliflozin

| S No. | HQC(1.9 µg/ml) | MQC(1.2 µg/ml) | LQC(0.18 µg/ml) | LLOQC (0.06 µg/ml) |
|-------|----------------|----------------|-----------------|--------------------|
|       | Concentration Found (µg/ml) |                      |                      |                    |                    |
| 1     | 1.915           | 1.181           | 0.177           | 0.058             |
| 2     | 1.917           | 1.194           | 0.182           | 0.062             |
| 3     | 1.913           | 1.203           | 0.173           | 0.052             |
| 4     | 1.911           | 1.185           | 0.170           | 0.056             |
| 5     | 1.922           | 1.198           | 0.175           | 0.059             |
| 6     | 1.912           | 1.189           | 0.171           | 0.063             |
| Mean Concentration (µg/ml) | 1.9150           | 1.1916          | 0.1746          | 0.0583            |
| SD    | 0.0041          | 0.0083          | 0.0045          | 0.0041            |
| %CV   | 0.21            | 0.69            | 2.53            | 6.91              |
| Mean Accuracy (%) | 99.74            | 99.31           | 97.04           | 97.22             |

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Table 4: With-in run Accuracy and Precision data for Canagliflozin

| S No | HQC (1.9 µg/ml) | MQC (1.2 µg/ml) | LQC (0.18 µg/ml) | LLOQC (0.06 µg/ml) |
|------|-----------------|-----------------|------------------|-------------------|
|      | Concentration Found (µg/ml) |                  |                  |                   |
| 1    | 1.910           | 1.198           | 0.172            | 0.057             |
| 2    | 1.915           | 1.195           | 0.180            | 0.058             |
| 3    | 1.920           | 1.189           | 0.173            | 0.063             |
| 4    | 1.908           | 1.193           | 0.175            | 0.062             |
| 5    | 1.922           | 1.196           | 0.174            | 0.055             |
| 6    | 1.918           | 1.194           | 0.182            | 0.059             |
| Mean | 1.9155          | 1.1941          | 0.1760           | 0.0590            |
| SD   | 0.0056          | 0.0031          | 0.0041           | 0.0031            |
| %CV  | 0.29            | 0.26            | 2.30             | 5.14              |
| Mean Accuracy | 99.77 | 99.51 | 97.78 | 98.33 |

Table 5: With-in run Accuracy and Precision data for Canagliflozin

| S No | HQC (1.9 µg/ml) | MQC (1.2 µg/ml) | LQC (0.18 µg/ml) | LLOQC (0.06 µg/ml) |
|------|-----------------|-----------------|------------------|-------------------|
|      | Concentration Found (µg/ml) |                  |                  |                   |
| 1    | 1.917           | 1.191           | 0.178            | 0.062             |
| 2    | 1.921           | 1.203           | 0.182            | 0.058             |
| 3    | 1.915           | 1.195           | 0.184            | 0.055             |
| 4    | 1.918           | 1.197           | 0.176            | 0.063             |
| 5    | 1.923           | 1.193           | 0.174            | 0.056             |
| 6    | 1.916           | 1.189           | 0.172            | 0.059             |
| Mean | 1.9183          | 1.1946          | 0.1776           | 0.0588            |
| SD   | 0.0031          | 0.0050          | 0.0047           | 0.0032            |
| %CV  | 0.16            | 0.42            | 2.61             | 5.42              |
| % Mean Accuracy | 99.91 | 99.91 | 98.70 | 98.06 |

Between Batch Accuracy and precision

| N    | 18              | 18              | 18               | 18                |
| Mean Concentration (µg/ml) | 1.9162          | 1.1935          | 1.7611           | 0.0587            |
| SD   | 0.0044          | 0.0057          | 0.0043           | 0.0033            |
| %CV  | 0.23            | 0.47            | 2.44             | 5.54              |
| % Mean Accuracy | 99.81 | 99.46 | 97.84 | 97.87 |
Preparation of Canagliños Stock solution (120 μg/ml) Accurately weighed and transferred 2 mg of Canagliños hemihydrate into a 200 mL volumetric flask, added 120 mL of diluent and sonicated for 5 minutes. Diluted up to the mark with diluent and mixed (120 μg/ml of Canagliños).

Preparation of Canagliños Spiking Solutions (0.06 μg/ml to 2.4 μg/ml) From the above Canagliños stock solution 0.1ml, 0.2ml, 0.3ml, 1.2ml, 2.0ml, 2.4ml, 3.2ml and 4.0 ml was transferred using micro pipette into 8 individual 20 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.6 μg/ml, 1.2 μg/ml, 1.8 μg/ml, 4.8 μg/ml, 12.0 μg/ml, 14.4 μg/ml, 19.2 μg/ml and 24 μg/ml.

Preparation of Calibration Standards and Quality control samples Calibration standards and quality control (QC) samples were made by mixing blank human plasma with Canagliños Spiking Solution to get 0.06 μg/ml, 0.12 μg/ml, 0.18 μg/ml, 0.48 μg/ml, 1.2 μg/ml, 1.44 μg/ml, 1.92 μg/ml and 2.4 μg/ml. (of Canagliños)

Preparation of internal standard Solution (5 μg/ml) Accurately weighed and transferred 12.5 mg of Dapagliños in to a 50 ml volumetric flask added 30 ml of diluent and sonicated for 5 minutes and

### Table 6: Between run Accuracy and Precision data for Canagliños

| S N | Change of HPLC Column ID | HQC (1.9 μg/ml) | MQC (1.2 μg/ml) | LQC (0.18 μg/ml) | LLOQC (0.06 μg/ml) |
|-----|--------------------------|----------------|----------------|-----------------|-------------------|
|     |                          | Concentration Found (μg/ml) |               |                 |                   |
| 1   | HPLC Column 2            | 1.917          | 1.196          | 0.176           | 0.056             |
| 2   |                          | 1.923          | 1.198          | 0.183           | 0.058             |
| 3   |                          | 1.916          | 1.203          | 0.177           | 0.062             |
| 4   |                          | 1.919          | 1.201          | 0.179           | 0.056             |
| 5   |                          | 1.920          | 1.197          | 0.181           | 0.060             |
| 6   |                          | 1.924          | 1.194          | 0.178           | 0.058             |
| N   |                          | 6              | 6              | 6               | 6                 |
| Mean Concentration (μg/ml) | 1.9198         | 1.1981         | 0.179          | 0.0583           |
| SD  |                          | 0.0032         | 0.0034         | 0.0027          | 0.0024            |
| % CV|                          | 0.17           | 0.28           | 1.46            | 4.01              |
| % Mean Accuracy             | 99.99          | 99.85          | 99.44          | 97.22             |

### Table 7: Between run Precision data for Canagliños.

| S No | Change of Analyst | HQC (1.9 μg/ml) | MQC (1.2 μg/ml) | LQC (0.18 μg/ml) | LLOQC (0.06 μg/ml) |
|------|-------------------|----------------|----------------|-----------------|-------------------|
|      |                   | Concentration Found (μg/ml) |               |                 |                   |
| 1    | Analyst-2         | 1.921          | 1.202          | 0.182           | 0.062             |
| 2    |                   | 1.917          | 1.197          | 0.177           | 0.058             |
| 3    |                   | 1.919          | 1.195          | 0.174           | 0.061             |
| 4    |                   | 1.923          | 1.204          | 0.183           | 0.057             |
| 5    |                   | 1.916          | 1.198          | 0.178           | 0.063             |
| 6    |                   | 1.918          | 1.196          | 0.182           | 0.059             |
| N    |                   | 6              | 6              | 6               | 6                 |
| Mean Concentration (μg/ml) | 1.919          | 1.1986         | 0.1793         | 0.060             |
| SD   |                   | 0.0027         | 0.0036         | 0.0036          | 0.0024            |
| % CV |                   | 0.14           | 0.30           | 1.98            | 3.94              |
| % Mean Accuracy              | 99.95          | 99.89          | 99.63          | 100.00           |
Table 8: Recovery- Study of Canagliftlozin from Human Plasma.

| S No | HQC (1.9 μg/ml) | MQC (1.2 μg/ml) | LQC (0.18 μg/ml) |
|------|-----------------|-----------------|------------------|
|      | Un-extracted    | Extracted       | Un-extracted     | Extracted       |
|      | area            | sample          | area             | sample          |
| 1    | 230567          | 213384          | 138082           | 135494          |
| 2    | 231516          | 213162          | 138498           | 136540          |
| 3    | 232044          | 212871          | 137951           | 135497          |
| 4    | 230181          | 212687          | 138808           | 134652          |
| 5    | 232393          | 213934          | 137521           | 135384          |
| 6    | 233095          | 217481          | 138134           | 134545          |
| N    | 6               | 6               | 6                | 6               |
| Mean Area | 231633          | 213920          | 138166           | 135352          |
| SD   | 1108.36         | 1797.97         | 445.58           | 720.39          |
| % CV | 0.48            | 0.84            | 0.32             | 0.53            |
| Mean Recovery (%) | 92.35           | 97.96           | 93.42            |

Overall Mean Recovery (%) 94.580
Overall SD 2.9787
Overall CV (%) 3.15

Table 9: The Matrix sample long term stability of Canagliftlozin at -28 ± 5°C (60 Days).

| S No | HQC (1.9 μg/mL) | LQC (0.18 μg/mL) |
|------|-----------------|------------------|
|      | Control Sample  | Stability sample | Control Sample  | Stability sample |
| 1    | 1.917           | 1.923            | 0.177           | 0.175            |
| 2    | 1.920           | 1.915            | 0.182           | 0.185            |
| 3    | 1.923           | 1.921            | 0.176           | 0.182            |
| 4    | 1.916           | 1.915            | 0.178           | 0.178            |
| 5    | 1.918           | 1.922            | 0.183           | 0.176            |
| 6    | 1.922           | 1.919            | 0.181           | 0.179            |
| Mean Concentration | 1.9193          | 1.9191          | 0.1795          | 0.1791           |
| SD   | 0.0029          | 0.0035           | 0.0029          | 0.0038           |
| % CV | 0.15            | 0.18             | 1.60            | 2.10             |
| Mean accuracy (%) | 99.97           | 99.96            | 99.72           | 99.54            |
| Mean stability (%) | 99.96           | 99.63            |

diluted up to the mark with diluent and mixed (250 μg/ml of Dapagliftlozin). Further diluted 1.0 ml of above solution to 10 ml with diluent and mixed (25 μg/ml of Dapagliftlozin).

NOTE: 0.5ml (500 μl) of the above internal standard solution is to be mixed with spiking blank plasma and working stock dilutions of analyte to get 5 μg/ml Dapagliftlozin (internal standard).

**Extraction procedure** Using a 1.0 ml micro pipette transferred 750 μL of human plasma and 500 μL of internal standard solution, 250 μL of Canagliftlozin spiking solutions into a 15 ml graduated dry centrifuge tube and vortexed for 30 sec. Added 1 ml of Acetonitrile into the above centrifugation tube and mixed. Vortexed the mixture for 3 min. Centrifuged the mixture in the centrifuge tube at 4200 rpm for 12 min. The supernatant liquid after the centrifugation was been filtered using 0.45 μ membrane filter.
Table 10: Matrix sample long term stability of Canagliftalin at -80 ± 5 °C (60 Days).

| S No | HQC (1.9 μg/mL) Control Sample | Stability sample | LQC (0.18 μg/mL) Control Sample | Stability sample |
|------|-------------------------------|-----------------|---------------------------------|-----------------|
| 1    | 1.923                         | 1.921           | 0.178                           | 0.182           |
| 2    | 1.915                         | 1.924           | 0.181                           | 0.184           |
| 3    | 1.918                         | 1.917           | 0.176                           | 0.185           |
| 4    | 1.922                         | 1.915           | 0.175                           | 0.177           |
| 5    | 1.919                         | 1.920           | 0.182                           | 0.181           |
| 6    | 1.917                         | 1.922           | 0.184                           | 0.178           |
| Mean | Concentration                 |                 | Mean stability (%)              |                 |
|      | 1.919                         | 1.919           | 0.1793                          | 0.1811          |
| SD   | 0.0031                        | 0.0034          | 0.0036                          | 0.0032          |
| % CV | 0.16                          | 0.17            | 1.98                            | 1.76            |
| %Mean accuracy | 99.95                | 99.99          | 99.63                           | 100.65          |
| Mean stability (%) | 99.97                |                | 100.14                          |                 |

RESULTS AND DISCUSSION

Chromatographic conditions development

Method finalization was performed on basis of retention time, USP Tailing factor, USP Resolution and peak areas found for Canagliftalin and that of internal standard. The mobile phase pH was finalized on the basis to shorten the run time (15 min) and different trials were performed during method optimization. Peak retention time of Canagliftalin and Dapagliftalin were found to be about 8.7 min and 4.1 min respectively. USP plate count for Dapagliftalin peak and Canagliftalin peak are found to be 3100 and 6500 respectively (from Medium QC (MQC) solution). USP tailing for Dapagliftalin peak and Canagliftalin peak are found to be 1.05 and 1.03 respectively (MQC solution). USP resolution between Dapagliftalin peak and Canagliftalin peak was found to be 13.7 (MQC solution). The reported method is with shorter run time and more number of aliquots can be analyzed in less time. Typical chromatogram of MQC solution obtained by optimized chromatographic condition is presented in Figure 2.

Method Validation (Bio-Analytical)

The developed RP-HPLC method for the estimation of Canagliftalin was validated by following ICH Guidelines.

System Suitability-Evaluation

Evaluation of System suitability parameters was performed by giving 6 replicate injections of MQC concentration. Evaluation of System suitability was performed on every day sequence as the first experiment. Results of system suitability evaluations are presented in Table 1.

Selectivity/Specificity

Specificity of the proposed method is validated by analyzing blank samples of the biological matrix (6 batches of di-potassium ethylene diamine tetra acetic acid blank plasma and extracted blank plasma). No peak was observed in the retention time of Canagliftalin (analyte peak) and that of Dapagliftalin (internal standard). A typical chromatogram of plasma extracted blank and chromatogram of internal standard are shown in Figures 3 and 4. Carryover was studied by injecting the blank sample after the sample (MQC) and no impact of carryover was found.

Linearity

The method linearity was demonstrated over the range of 0.06 to 2.4 μg /ml for Canagliftalin. The coefficient of correlation (R²) value was found to be 0.999 (Limit: not less than 0.99). The relationship adequately describes the response of the instrument with that of concentration of Canagliftalin. The slope and y-intercept of the calibration curve were 0.108 and 0.001 respectively. A specimen calibration curve obtained during the precision and accuracy study of validation is presented in Figure 5. The back calculated nominal concentrations obtained for calibration standard are presented in Table 2 along with the mean calculated accuracy value.

Precision and Accuracy

Within day and between day precision and accuracy were studied by determining 6 replicates of Lower Limit of Quantitation (LLOQC), Low (LQC), MQC and High (HQC). Accuracy and Precision method validation parameter were evaluated by 6 replicate analysis for Canagliftalin at 4 concentration levels, i.e., 0.06 μg/ml (LLOQC), 0.18 μg/ml (LQC), 1.2
µg/ml (MQC) and 1.9 µg/ml (HQC). Within-day and between day accuracy of plasma samples were studied and excellent mean % accuracy were obtained with range varied from 97.84 % to 99.81 % for within day and 97.22 % to 100.00 % for between day respectively. Precision of canagliθlozin in plasma samples were studied and found to be 0.23 % to 5.54 % for within day and 0.14 % to 4.01 % for between days respectively (Tables 3, 4, 5, 6 and 7).

Recovery

Recovery studies are performed by measuring the peak areas found for Canagliθlozin and that of internal standard, by comparing the results of extracted sample with corresponding extracts of blank spiked with Canagliθlozin post-extraction. Accuracy values obtained for Canagliθlozin at LQC, MQC and HQC were found to be 93.42 %, 97.96 % and 92.35% respectively (Table 8). Overall average accuracy of Dapagliθlozin (Internal Standard) was found to be 98.07%.

Solution Stability

Short term stock solution stability

During bench-top solution stability studies, 6 replicates of LQC & HQC samples (0.12 and 3.2 µg/ml) were analyzed after 9 hours at controlled room temperature on the laboratory bench (about 25°C). The mean stability of solution was determined and found to be 99.72 % for LQC and 99.99 % for HQC respectively.

Long term Matrix sample Solution stability at -28±5 oC & -80±5 oC for 60 Days

Long term matrix stock solution stability for the Canagliθlozin was studied at a concentration of LQC-HQC level after a storage period of about 60 days at -28°C & -80°C in refrigerator. The mean solution stability of Canagliθlozin was 99.63 %, 99.96 % & 100.14 % and 99.97 % (Tables 9 and 10).

CONCLUSIONS

The novel method validated as per ICH guidelines met with the preset acceptance criterias. This novel bio-analytical method can be used for pharmacokinetic studies in the clinical laboratories.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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