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# Validation of Stability-Indicating Reverse Phase HPLC Method For The Determination of Related Substances in Canagliflozin Drug Substance

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***Abstract:*** *A gradient reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination for related substances of Canagliflozin drug substance. Good chromatographic separation of Canagliflozin from its process and degradation related substances was achieved on Ascentis Express RP-Amide, 150mm × 4.6mm 2.7µm i.e stainless steel column 150 mm long, 4.6 mm internal diameter filled with amide groups chemically bonded to porous silica particles of 2.7 µm diameter maintained column oven temperature at 30°C. Ammonium acetate buffer is mobile phase A and acetonitrile is mobile phase B. Wavelength for UV detection: 290nm, flow rate: 0.7 ml/min and Injection volume: 10µl. The method suitability checked and validated according to the ICH guidelines for specificity, linearity, accuracy, precision, limit of quantification, limit of detection, robustness and ruggedness and also Canagliflozin was subjected to stress conditions of thermal, hydrolysis, humidity, peroxide and photolytic to observe the degradation products. Limit of detection of each RS is less than 0.008%w/w indicating that the developed method is highly sensitive. The experiment results are given in detailed in this research article.*

***Keywords:*** *Canagliflozin, Related substances, HPLC, Validation*

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## I. Introduction

Canagliflozin is chemically known as (2*S*,3*R*,4*R*,5*S*,6*R*)-2-[3-[[5-(4-fluorophenyl)thiophen-2-yl] methyl]-4-methylphenyl]tetrahydro-6-hydroxymethyl-2*H*-pyran-3,4,5-triol, molecular formula is $C_{24}H_{25}FO_5S$ and molecular weight is 444.52. Canagliflozin is an oral selective Sodium-Glucose co-transporter 2 (SGLT2) inhibitor used for the management of type 2 Diabetes Mellitus [1]. It curbs the transporter protein SGLT2 present in the proximal tubules of the kidney which curtails renal glucose absorption, thereby increasing urinary glucose excretion and lowering blood glucose levels [2-3]. SGLT2 inhibitors are a promising new class of antidiabetic drugs currently approved in Europe, the US and Japan. They allow weight-reducing and effective glycaemic control combined with a low risk of hypoglycaemia [4]. The recommended starting dose is 100 mg once daily, taken before the first meal of the day (2.1). Dose can be increased to 300 mg once daily and this dosage is approved by FDA [5].Canagliflozin is marketed under trade name INVOKANA [5] , manufactured by Janssen Pharmaceuticals, Inc. Titusville, NJ 08560 and Licensed from Mitsubishi Tanabe Pharma Corporation , Japan, supplied as film-coated tablets for oral administration, containing 102 and 306 mg of Canagliflozin in each tablet strength, corresponding to 100 mg and 300 mg of Canagliflozin (anhydrous), respectively. The chemical structure of Canagliflozin is shown in Figure 1.

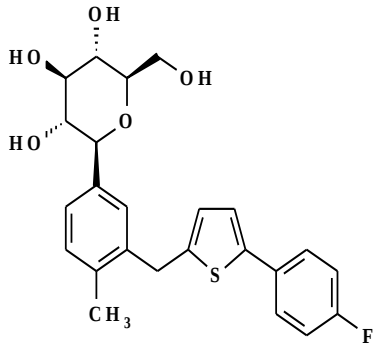


**Figure 1:** Chemical structure of Canagliflozin

There is no pharmacopoeia monograph available for this drug substance or drug product and no HPLC method is available in literature for quantification of Canagliflozin related substances. However, few methods have been reported in literature for the determination of Canagliflozin in formulated products. As per the available literature, it is revealed that the drug has been estimated by Liquid chromatography in Metformin hydrochloride and Canagliflozin oral tablets formulations [6], determination of Canagliflozin in plasma samples by HPLC [7], estimation of canagliflozin in dosage form by HPLC [8] and UV Spectrometric method [9]. In this research paper, development of a stability indicating HPLC method for the simultaneous detection and quantitative determination of the four related substances in canagliflozin drug substance has been reported. The chemical structures of these four related substances [RS –I to IV] are given in Figure 2. Forced degradation studies were carried out to establish stability indicating nature of the method according with ICH stability guidelines [10]. Limit of detection (LOD), limit of quantification (LOQ) and linearity were established as per ICH guidelines. The limit of these impurities has been considered as 0.15% in accordance with ICH guideline based on maximum daily dose [11]. The developed chromatographic method can resolve four related substances with acceptable resolution to achieve good chromatography and the optimized methodology have been validated to accomplish ICH guidelines on validation [12]. The same method can also be utilized for the determination of assay of canagliflozin drug substance.

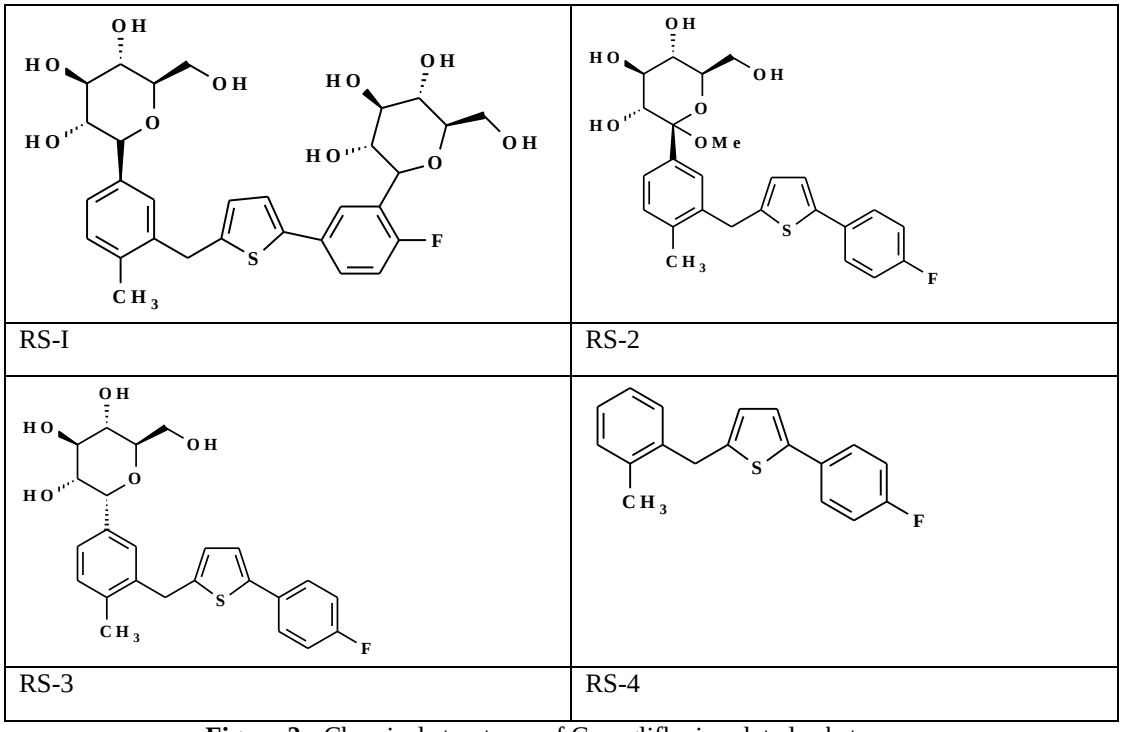



**Figure 2:** Chemical structures of Canagliflozin related substances

## II. Materials And Methods

### 2.1 Chemicals, reagents, standards and samples

The investigated samples of Canagliflozin drug substance and reference sample, its related substances and Canagliflozin for system suitability (Canagliflozin enriched with RS-2) were gifted from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). AR grade of ammonium acetate, glacial acetic acid (GR grade), acetonitrile (HPLC grade) and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA). Hydrochloric acid (~35%), Sodium hydroxide (GR grade) and Hydrogen peroxide (~30 %) are used for forced degradation experiments under method validation.

### 2.2 Instrumentation and methodology

The HPLC systems used for method development, method validations as well as for forced degradation studies were Waters Alliance e2695 separation module equipped with 2489 UV detector, waters 2695 separation module with 2996 PDA detector with Empower data handling system i.e Empower 3 software, [Waters Corporation, MILFORD, MA 01757, USA]. HPLC column: Analytical column: A stainless steel column 150 mm long, 4.6 mm internal diameter filled with amide groups chemically bonded to porous silica particles of 2.7 µm diameter. [Ascentis Express RP-Amide, 150mm X 4.6mm, 2.7µm, Make : Supelco], column oven temperature: 30°C. Mobile phase A : pH 6.5 Buffer (Dissolve 0.77 g of ammonium acetate in 1000 ml of water. Adjust pH to 6.5±0.05 with dilute glacial acetic acid solution(i.e Dilute 2 ml of glacial acetic acid to 100 ml with water.). Filter through 0.45 µ or finer porosity membrane filter), Mobile phase B: Acetonitrile. Diluent: degassed mixture of water and acetonitrile in the ratio of 50:50 v/v. Flow rate: 0.7 ml/min, injection volume: 10µl, UV detection: 290nm, data acquisition time: 65 min. Retention time of Canagliflozin is 23 min. The pump is in gradient mode and the program is as follows: Time (min)/ A (v/v): B (v/v); $T_{0.01}$/75:25, $T_{30}$/55:45, $T_{45}$/25:75, $T_{55}$/05:95, $T_{65}$/5:95, $T_{67}$/75:25.,$T_{75}$/75:25.

### 2.3 Preparation of solutions

**2.3.1** System suitability solution

0.5 mg/ml concentration of Canagliflozin for system suitability (Canagliflozin enriched with RS-2) in diluent.

**2.3.2 Standard solution**

0.00075 mg/ml concentration of solution using Canagliflozin standard in diluent. [i.e 50 mg of Canagliflozin working standard into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve. Make up to volume with diluent. Dilute 5 ml to 100 ml with diluent. Further dilute 3 ml of this solution to 100 ml with diluent.]

**2.3.3 Sample solution**

0.5 mg/ml concentration of solution using Canagliflozin sample in diluent [50 mg of sample into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve. Make up to volume with diluent].

**2.3.4 System suitability evaluation**

USP resolution between Canagliflozin and RS-2 is not less than 3.0.

## III. Results And Discussion

### 3.1 Method validation

The developed method was established through the validation experiments as per the ICH guidelines individually in terms of specificity or selectivity, forced degradation studies, LOD/LOQ, linearity, precision, accuracy, robustness and stability of solutions.

### 3.2 Specificity

Specificity is the ability of assess unequivocally of analytic in the presence of components which may be expected to be present and prove the capability of method performance. For determination of specificity, blank, all individual related substances solutions were prepared and injected to confirm the individual retention times. The solutions of Canagliflozin drug substance (Control Sample) and Canagliflozin spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. A typical representative HPLC chromatogram of Canagliflozin drug substance spiked with all related substances is shown in Figure 3. The specificity results are tabulated in Table 1.

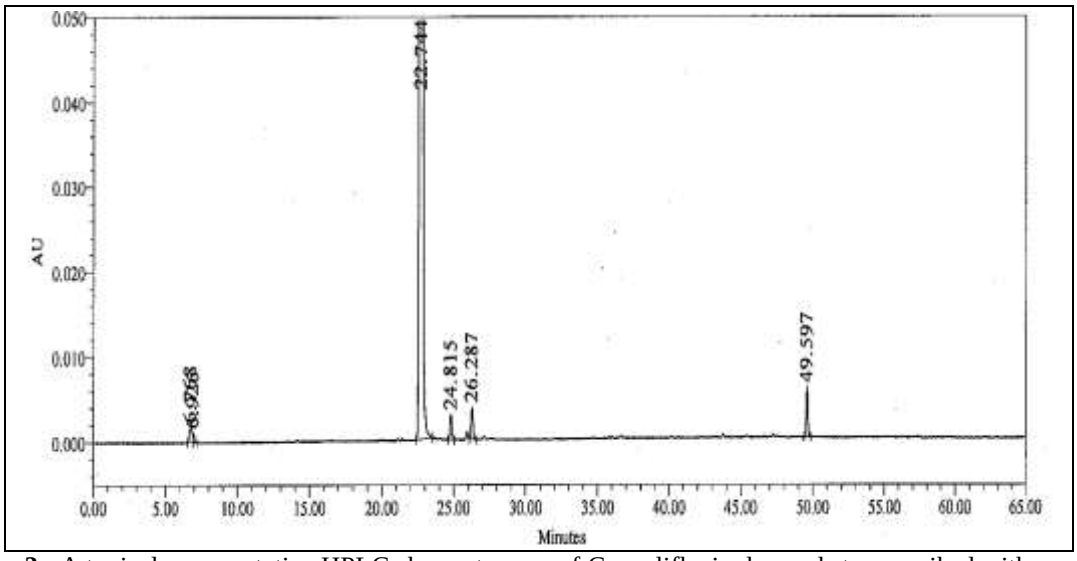



**Figure 3:** A typical representative HPLC chromatogram of Canagliflozin drug substance spiked with related substances

**Table 1**: Specificity experiments results

| Name | Retention time (min) | RRT | Peak purity | |
|---|---|---|---|---|
| | | | Purity angle | Purity threshold |
| RS-1 (Isomer-1) | 6.768 | 0.30 | 0.792 | 1.673 |
| RS-1 (Isomer-2) | 6.926 | 0.30 | 1.266 | 3.267 |
| Canagliflozin | 22.744 | 1.00 | 0.082 | 0.602 |
| RS-2 | 24.815 | 1.09 | 0.845 | 1.555 |
| RS-3 | 26.287 | 1.16 | 0.729 | 1.307 |
| RS-4 | 49.597 | 2.18 | 0.507 | 0.951 |

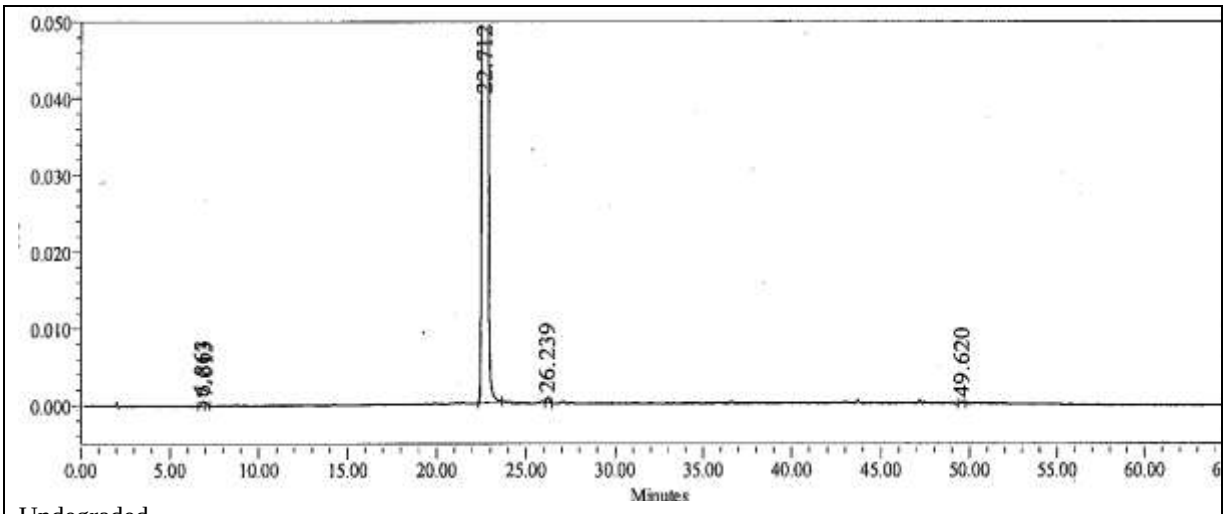
Specificity experiment reveals that no peak was observed at the retention time of Canagliflozin and its known related substances in the chromatogram of diluent. From the specificity chromatograms of all known related substances and spiked sample indicates that the related substances are well separated from closely eluting peaks of other known related substances and Canagliflozin. The peak purity data of Canagliflozin peak in control sample and spiked sample, known related substances peaks in spiked sample showed that the peaks are homogeneous and have no co-eluting peaks. Based on this information, it can be concluded that the test method is specific for the determination of related substances in Canagliflozin drug substance.

### 3.1.2 Forced degradation

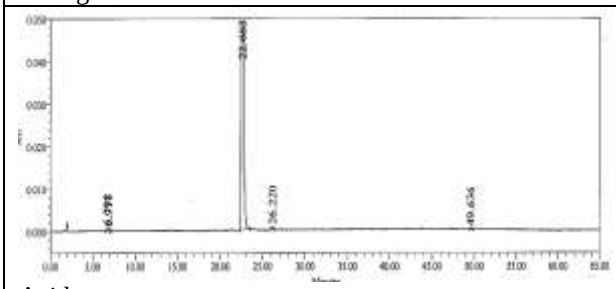
The stability indicating nature of Canagliflozin drug substance has been studied by proving forced degradation study experiments. Canagliflozin was subjected to different stress conditions i.e acid/base hydrolysis [5M HCl/85°C/180 min & 5M NaOH/85°C/180 min], peroxide degradation under oxidative stress [30% $H_2O_2$ / 85°C / 120 min], thermal degradation [65°C/120 Hours], humidity degradation study (92.5% RH/25°C/120 hrs) and photolytic degradation [white Fluorescent light, 1.2million lux hours and UV light, 200 watt hours / $m^2$] w.r.t ICH option 2 of Q1B [13]. Peak purity of Canagliflozin peak was established by using PDA detector in these stress samples along with unstressed sample. The forced degradation results are tabulated in Table 2. There was no significant change observed w.r.t all known impurities in forced degradation studies. Two unknown impurities were formed at RRT~0.74 & RRT~0.76 to the level of 0.15% & 0.15% respectively in peroxide degradation condition. In photolytic degradation, many of unknown impurities were formed, but some of detected at higher levels [i.e 0.32% (RRT 0.07), 0.28% (RRT 0.09), 0.25%(RRT 0.76), 1.58%(RRT 1.34), 0.34%, 0.50% (RRT 1.67)].The results of forced degradation studies indicated that Canagliflozin is susceptible to degradation under photolytic stress condition whereas, it is found to be stable in acidic, basic, oxidative hydrolytic conditions, humidity and thermal stress conditions. Further, the evaluation of peak purity Canagliflozin peak from the analysis of every degradation sample showed that it is homogeneous, and there are no co-eluting peaks. Based on this information, the test method is declared to be specific and stability indicating for the specified / unspecified related substances in Canagliflozin drug substance. The forced degradation experiments results are shown in Table 2 and typical HPLC chromatograms are shown in Fig.4.

**Table 2:** Canagliflozin Forced degradation experiments data

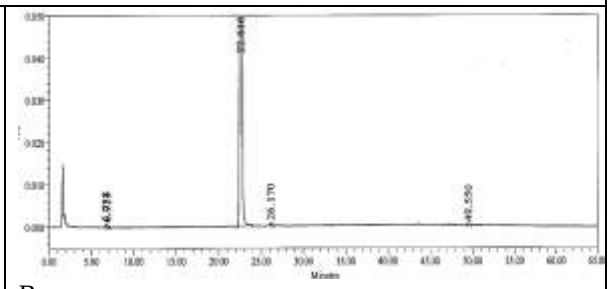
| Degradation mechanism | Degradation condition | Area | Area degradation (%) | Peak purity of Canagliflozin | |
|---|---|---|---|---|---|
| | | | | Purity angle | Purity threshold |
| Undegraded | Unstressed sample | 15903505 | -- | 0.032 | 0.348 |
| Acid | 5M HCl / 85°C / 180 min | 16043871 | Nil | 0.031 | 0.319 |
| Base | 5M NaOH / 85°C / 180 min | 15893828 | 0.1 | 0.047 | 0.332 |
| Peroxide | 30% $H_2O_2$ / 85°C / 120 min | 15228888 | 0.7 | 0.010 | 0.395 |
| Thermal | 65°C / 120 hours | 15804558 | 0.9 | 0.022 | 0.275 |
| Photolytic | White fluorescent light, 1.2million Lux hours and UV light, 200 watt hours / meter square | 13559357 | 14.0 | 0.023 | 0.307 |
| Humidity | 92.5% RH / 25°C / 120 hours | 15852674 | 0.6 | 0.023 | 0.273 |



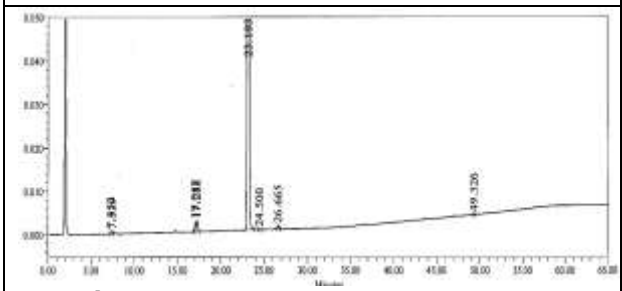

Undegraded



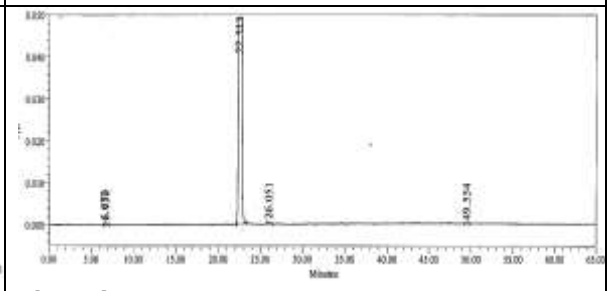
Acid



Base



Peroxide



Thermal

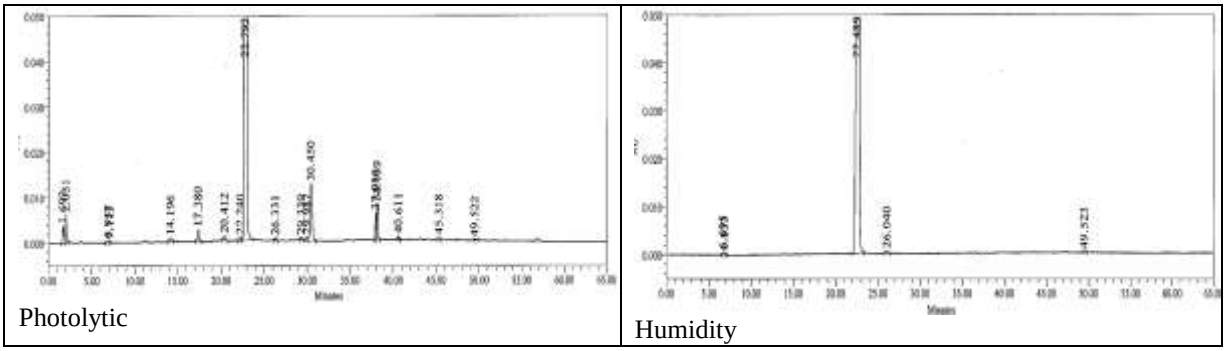



**Fig. 4:** Typical representative HPLC chromatograms of Canagliflozin drug substance -Forced degradation experiments

### 3.1.3 Limit of Detection (LOD) / Limit of Quantification (LOQ)

LOD and LOQ were predicted on the basis of response and slope of the regression equation. These values are calculated from the formula 3.3δ/S and 10 δ /S respectively where 'δ is standard deviation of the y-intercept of the regression line and 'S' is slope of the calibration curve which were obtained from linearity experiments carried out from 5% to 150% specification levels for each RS . The precision study was carried out at about predicted LOD and LOQ levels by injecting six replicates and calculating the % RSD of the area of each RS. LOD and LOQ values are presented in Table 3.

### 3.1.4 Linearity

A series of solutions were prepared by using Canagliflozin and its related substances at concentration levels from 5 to 150% of specification level and each solution was injected and calculated the statistical values like slope, intercept, STEYX and correlation coefficient from linearity plot drawn for concentration versus area. From this data after establishing LOQ level for all the related substances including drug substance, the linearity has been deduced from LOQ level to 150% specification level and statistical values are presented in Table 3.

**Table 3:** Linearity and LOD/LOQ experiments

| | **RS-1** | **RS-2** | **RS-3** | **RS-4** | **Canagliflozin** |
|---|---|---|---|---|---|
| **Concentration range (µg/mL)** | 0.122-1.115 | 0.078-1.139 | 0.089-1.125 | 0.055-1.177 | 0.055-1.177 |
| **Slope** | 27071 | 30724 | 28372 | 50059 | 32489 |
| **Intercept** | -154 | 17 | 64 | 51 | 541 |
| **STEYX** | 203 | 90 | 52 | 88 | 215 |
| **Response factor** | 1.20 | 1.06 | 1.15 | 0.65 | 1.0 |
| **Correlation Coefficient** | 0.9998 | 0.9999 | 0.9999 | 0.9999 | 0.9998 |
| **LOD(%w/w)** | 0.008 | 0.005 | 0.006 | 0.004 | 0.005 |
| **%RSD** | 2.9 | 1.8 | 1.1 | 0.3 | 3.1 |
| **LOQ(%w/w)** | 0.024 | 0.016 | 0.018 | 0.011 | 0.015 |
| **%RSD** | 0.4 | 0.6 | 0.5 | 0.4 | 9.6 |

### 3.1.5 Precision

The system precision was evaluated by injecting five injections of Canagliflozin standard solution at a concentration of 0.5 mg/ml and calculating the % relative standard deviation (% RSD). The method precision was checked by injecting six individual preparations of Canagliflozin drug substance spiked with RS-1, RS-2, RS-3 and RS-4 with 0.15% level, % RSD of content of each related substance was calculated. The intermediate precision of the method was also evaluated using different analyst, different instrument, and different lot of column on different day. The inter day variations

**Table 4a:** System Precision experiment results

| **Injection ID** | **Canagliflozin peak area** |
|---|---|
| Injection-1 | 15477783 |
| Injection-2 | 15488153 |
| Injection-3 | 15506169 |
| Injection-4 | 15547814 |
| Injection-5 | 15490584 |
| **Mean** | 15502101 |
| **SD** | 27499 |
| **%RSD** | 0.18 |
| **95% Confidence Interval (±)** | 28863 |

were calculated. The precision experiments results are given in Table 4. The results (method precision and intermediate precision) indicated that the method is rugged for the determination of related substances in Canagliflozin drug substance w.r.t analyst-to-analyst, system-to-system, column-to-column and day-to-day variations.

**Table 4b:** Method Precision experiment results

| | **Inj-1** | **Inj-2** | **Inj-3** | **Inj-4** | **Inj-5** | **Inj-6** | **Mean (%w/w) *n=6*** | **SD** | **% RSD** | **95% Confidence Interval (±)** |
|---|---|---|---|---|---|---|---|---|---|---|
| RS-1 | 0.237 | 0.238 | 0.237 | 0.238 | 0.239 | 0.238 | 0.238 | 0.001 | 0.4 | 0.001 |
| RS-2 | 0.150 | 0.151 | 0.151 | 0.151 | 0.152 | 0.151 | 0.151 | 0.001 | 0.7 | 0.001 |
| RS-3 | 0.214 | 0.214 | 0.214 | 0.214 | 0.215 | 0.214 | 0.214 | 0.000 | 0.0 | 0.000 |
| RS-4 | 0.162 | 0.162 | 0.162 | 0.162 | 0.163 | 0.162 | 0.162 | 0.000 | 0.0 | 0.000 |

**Table 4c :** Intermediate Precision experiment results

| | **Inj-1** | **Inj-2** | **Inj-3** | **Inj-4** | **Inj-5** | **Inj-6** | **Mean (%w/w) *n=6*** | **SD** | **% RSD** | **95% Confidence Interval (±)** |
|---|---|---|---|---|---|---|---|---|---|---|
| RS-1 | 0.222 | 0.226 | 0.226 | 0.226 | 0.225 | 0.225 | 0.225 | 0.002 | 0.9 | 0.002 |
| RS-2 | 0.145 | 0.145 | 0.144 | 0.144 | 0.145 | 0.146 | 0.145 | 0.001 | 0.7 | 0.001 |
| RS-3 | 0.208 | 0.208 | 0.206 | 0.207 | 0.208 | 0.207 | 0.207 | 0.001 | 0.5 | 0.001 |
| RS-4 | 0.160 | 0.159 | 0.158 | 0.159 | 0.159 | 0.158 | 0.159 | 0.001 | 0.6 | 0.001 |

**Table 4d :** Cumulative results of precision experiments

| | | **Inj-1** | **Inj-2** | **Inj-3** | **Inj-4** | **Inj-5** | **Inj-6** |
|---|---|---|---|---|---|---|---|
| **Set-1(for method precision)** | RS-1 | 0.237 | 0.238 | 0.237 | 0.238 | 0.239 | 0.238 |
| | RS-2 | 0.150 | 0.151 | 0.151 | 0.151 | 0.152 | 0.151 |
| | RS-3 | 0.214 | 0.214 | 0.214 | 0.214 | 0.215 | 0.214 |
| | RS-4 | 0.162 | 0.162 | 0.162 | 0.162 | 0.163 | 0.162 |
| **Set-2 (for intermediate precision)** | RS-1 | 0.222 | 0.226 | 0.226 | 0.226 | 0.225 | 0.225 |
| | RS-2 | 0.145 | 0.145 | 0.144 | 0.144 | 0.145 | 0.146 |
| | RS-3 | 0.208 | 0.208 | 0.206 | 0.207 | 0.208 | 0.207 |
| | RS-4 | 0.160 | 0.159 | 0.158 | 0.159 | 0.159 | 0.158 |
| **Overall** | | **Mean** | **SD** | | **% RSD** | **95% confidence interval (±)** | |
| | RS-1 | 0.231 | 0.007 | | 3.0 | 0.004 | |
| | RS-2 | 0.148 | 0.003 | | 2.0 | 0.002 | |
| | RS-3 | 0.211 | 0.004 | | 1.9 | 0.003 | |
| | RS-4 | 0.161 | 0.002 | | 1.2 | 0.001 | |

### 3.1.6 Accuracy

The accuracy of the method was determined by analyzing Canagliflozin (n=3) samples spiked with four related substances at different levels (LOQ, 50%, 100% and 150% of specification levels). The percentage recovery values for all the impurities are calculated and tabulated in Table.5. The recovery results indicated that the test method has an acceptable level of accuracy for the determination of related substances in Canagliflozin drug substance from LOQ to 150% of specification.

**Table 5:** Accuracy experiment results

| **Recovery details (average 3 replicates)** | | RS-1 | RS-2 | RS-3 | RS-4 |
|---|---|---|---|---|---|
| -- | % Level | | | | |
| **Added (%w/w)** | LOQ | 0.0243 | 0.0156 | 0.0176 | 0.0107 |
| | 50 | 0.075 | 0.075 | 0.075 | 0.076 |
| | 100 | 0.150 | 0.150 | 0.151 | 0.153 |
| | 150 | 0.224 | 0.224 | 0.226 | 0.228 |
| **Recovered (%w/w)** | LOQ | 0.0234 | 0.0147 | 0.0181 | 0.0107 |
| | 50 | 0.073 | 0.070 | 0.077 | 0.073 |
| | 100 | 0.150 | 0.142 | 0.157 | 0.148 |
| | 150 | 0.224 | 0.212 | 0.233 | 0.221 |
| **Recovery (%)** | LOQ | 96.3 | 94.2 | 102.8 | 100.0 |
| | 50 | 97.8 | 93.3 | 102.7 | 96.1 |
| | 100 | 100.2 | 94.7 | 104.0 | 96.7 |
| | 150 | 100.0 | 94.6 | 103.1 | 96.9 |

### 3.1.7 Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and to evaluate system suitability requirement as per methodology. For this evaluation, system suitability solution and sample solution spiked with impurities at specification level were prepared as per test method and injected into HPLC. To study the effect of flow rate, ±10% variation of flow rate was studied. The effect of column temperature was studied by keeping 20°C and 30°C instead of 25°C. In the same manner, detection wavelength (±3 nm), variation in pH (±0.2 units) and organic in mobile phase (±2% absolute in Gradient Composition) have been verified and the results obtained from these experiments are summarized in Table 6.

**Table 6:** Robustness experiment results

| **Condition** | **Variation** | **System Suitability** | | **Spiked Sample (RRT)** | | | | |
|---|---|---|---|---|---|---|---|---|
| | | USP resolution | USP tailing | RS-1 (Isomer-1) | RS-1 (Isomer-2) | RS-2 | RS-3 | RS-4 |
| Original Method parameters | - | 8.9 | 0.9 | 0.30 | 0.30 | 1.09 | 1.16 | 2.18 |
| Flow | -10% | 8.5 | 0.9 | 0.31 | 0.32 | 1.09 | 1.15 | 2.09 |
| | +10% | 9.2 | 0.9 | 0.29 | 0.29 | 1.09 | 1.16 | 2.22 |
| Wavelength | -3 nm | 8.9 | 0.9 | 0.30 | 0.30 | 1.09 | 1.16 | 2.18 |
| | +3 nm | 8.9 | 0.9 | 0.30 | 0.30 | 1.09 | 1.16 | 2.18 |
| % Organic in gradient variation | -2% absolute | 9.0 | 0.9 | 0.35 | 0.36 | 1.08 | 1.14 | 1.97 |
| | +2% absolute | 6.6 | 1.0 | 0.26 | 0.26 | 1.10 | 1.18 | 2.45 |
| Column Oven Temperature | -5°C | 8.4 | 0.9 | 0.30 | 0.31 | 1.09 | 1.16 | 2.18 |
| | +5°C | 9.4 | 0.9 | 0.29 | 0.30 | 1.10 | 1.15 | 2.18 |
| pH Variation | - 0.2 units | 4.7 | 1.0 | 0.30 | 0.31 | 1.09 | 1.16 | 2.17 |
| | + 0.2 units | 4.3 | 1.1 | 0.30 | 0.31 | 1.09 | 1.16 | 2.17 |

The HPLC chromatograms of Canagliflozin drug substance spiked with known related substances at specification level obtained from each of the above varied conditions indicated that RRT's of related substances are comparable to that obtained from STP condition, however significant retention time (min) variation was observed in gradient variation (± 2% absolute) condition. Hence, it is recommended to maintain the gradient programme accurately as per the test procedure.

### 3.1.8 Stability of solutions

Standard solution and sample solution spiked with related substances at specification level were prepared and analysed initially and different time intervals by keeping the solutions at 25°C±2°C and at 5°C±3°C. Based on experimental data, the standard solution is stable up to 1440 mins (24 hours) at 25°C±2°C and sample solution is stable up to 1200 mins (20 hours) at 5°C±3°C.

## IV. Conclusion

A reverse phase stability indicating HPLC method was developed and validated for the quantitative determination of related substances of Canagliflozin drug substance. The present research work will help the manufacturers and suppliers of Canagliflozin to quantify the quality in terms of purity based on experimental results. Thus, it can be used for routine analysis, quality control and for determining quality during the stability studies of pharmaceutical analysis.

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