New Stability Indicating LC Method for Novel Antidiabetic Drug Canagliflozin Hemihydrate Quantification; Development and Validation

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ABSTRACT

Introduction: The present research includes a new RP-HPLC method development and validation of Canagliflozin Hemihydrate (CANAG) a novel antidiabetic drug. The novel analytical method developed was employed for the quantification of CANAG in bulk drug and as an Active Pharmaceutical Ingredient (API) in tablet formulation. **Methods:** The present study utilized HPLC instrument (Shimadzu) comprises of ultraviolet detector and COS-MICSIL 100 C_{18} (250 × 4.6 mm) 5 μm column. The mobile phase consists of acetonitrile: water (70:30%v/v) of pH 3.0±0.05 adjusted by addition of ortho-phosphoric acid. The chromatographic conditions were, flow rate 1 ml/min, run time 6.0 min, injection volume 20 μl and detection wavelength 282 nm at room temperature. The developed method was validated as per International Conference on Harmonization (ICH) guidelines for achieving robustness and accuracy. Forced degradation studies were carried out to prove the capability of the developed method to quantify the CANAG analyte response in presence of degraded products. **Results:** The presently developed novel analytical method possesses a shorter retention time 4.1 min. The results of validation parameters suggested that the presently developed

method was robust and accurate since %RSD, theoretical plates and tailing factor values were within the limits of ICH guidelines. The predominant principle peak was observed in the chromatogram for CANAG without any interference from peaks of the degraded products. **Conclusion:** The present research study concluded that the developed analytical method for CANAG was novel, robust accurate and the principle peak of CANAG was not affected by presence of degraded and products. The same method can be employed for analysis of CANAG as a bulk drug and as API in formulation.

Key words: Canagliflozin, Liquid Chromatography, Stability Indicating, Validation.

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DOI : 10.5530/phm.2019.2.11

INTRODUCTION

As per ICH guidelines the analytical method employed for the estimation of bulk drug and API in formulation must be validated to achieve robustness and accuracy of the method. The major recommendation and requirement of any analytical method developed for quantification must also be capable of quantifying the drug in presence of minute quantities of degraded products. Hence the developed novel methods of analysis must be a stability indicating in nature. In the present research work the attempt was made to develop and validate a novel RP-HPLC method with stability indicating property for CANAG and same method adopted to quantify CANAG as bulk drug and API in formulation.

Canagliflozin hemihydrate (CANAG) is an antidiabetic drug that chemically known as (1S)-1,5-Anhydro-1-C-[3-[[5-(4-fluorophenyl)-2-thienyl]methyl]-4-methylphenyl]-D-glucitol (Figure 1) and is the drug of the gliflozin class or subtype 2 sodium-glucose transport (SGLT-2) inhibitors used for the treatment of type 2 diabetes. CANAG is an inhibitor of subtype 2 sodium-glucose transport proteins (SGLT2), which is responsible for at least 90% of renal glucose reabsorption. After thorough exploration of available literature for Reverse Phase High Performance Liquid Chromatography (RP-HPLC), High Performance Thin Layer Chromatography (HPTLC) and UV-visible spectrophotometry methods,[1-3] we found that a minimum number of methods available for quantification of CANAG. The existing method available utilized formic acid as one of the components in mobile phase and it has volatile nature accounts to variation in composition of mobile phase[4] and the other researcher developed a method with a linearity range of 75 to 450 μg/ml, this method may not be suitable for quantification of CANAG at lower concentration.[5] One more method was developed and available for quantifying related substances for CANAG as bulk drug.[6]

In the present research study the attempt was made to develop a novel validated analytical method for the quantification of CANAG as bulk drug and as API in formulation. The linearity range was established at lower concentration. The developed method was economical since the method has shorter retention time attributed to more turnout of sample which would be an important requirement of present industry. As the requirement of ICH guidelines the developed novel analytical method was validated on the basis of parameters like Specificity, Precision, Linearity, Accuracy, Ruggedness, Robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ).[7-9] The drug CANAG was subjected to forced degradation to analyse the interference of degraded products on principle peak of CANAG.[10-14]

MATERIALS AND METHODS

Materials

A reference standard of CANAG (Potency: 100.4% w/w on anhydrous basis) procured from Watson laboratories, Mumbai and characterized by FTIR and UV spectroscopy. CANAG tablets (Invokana) were purchased from local pharmacy. The HPLC solvents used were of E-Merck (India) Ltd. Mumbai. Methanol, acetonitrile, ortho phosphoric acid (Merck, Mumbai, India) were used in the analysis. HPLC grade water was prepared using Millipore purification system.

Instruments

Electronic balance (Mettler Toledo), sonicator (Lab India), digital pH meter (systronics), hot air oven (Servewell), HPLC instrument (Shimadzu) consisting of ultraviolet detector equipped with LC solution software, Fourier-Transform Infra-Red spectroscopy (Shimadzu IR-1S
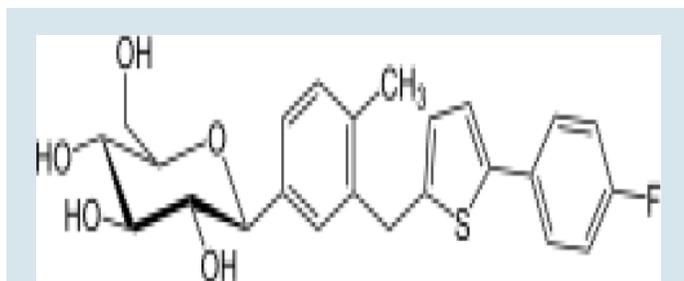
Figure 1: Structure of CANAG.

affinity), Ultra Violet Visible spectroscopy (Shimadzu-UV 1800).

Chromatographic Conditions

An HPLC instrument (Shimadzu) consisting of ultraviolet detector equipped with LC solution software with 282 nm used for the analysis. The chromatographic separations were performed on a COSMICSIL 100 C_{18} (250 × 4.6 mm), 5 μm column by keeping it in room temperature using a flow rate of 1.0 ml/min with run time 6.0 min. Injection volume was set as 20 μl.

Mobile Phase and diluent

The mobile phase was a mixture of Acetonitrile and Water (70:30%v/v), filtered through 0.2 μm finer porosity nylon membrane filter and degassed prior to use, adjusting the pH to 3.0±0.05 with ortho phosphoric acid. Mobile phase is also used as the diluent.

Standard Preparations

Standard stock solution was prepared by dissolving CANAG standard equivalent to 100 mg of canagliflozin hemihydrate in 100 ml of diluent. The above stock solution was diluted to get a final standard concentration of 100 μg/ml.

Sample Solution

Twenty tablets containing 100 mg of CANAG were weighed, average weight found and finely powdered. The sample solution was prepared by taking weight equivalent to 100 mg Canagliflozin from powdered twenty tablets into 100 ml volumetric flask and kept for sonication with 75 ml methanol for 30 min with intermittent shaking. Allowed to cool to room temperature and made up the volume with methanol. 5 ml of solution was diluted to 50 ml with mobile phase. Then the solution was filtered through 0.2 μm nylon membrane filter.

Validation[7-9]

The developed method has been validated for the assay of CANAG in formulation, 100 mg tablet strength using following parameters.

System Suitability

Six replicates of prepared 100 μg/ml concentration of CANAG standard were injected. From standard preparation analysis by the developed method the tailing factor obtained for CANAG peak should be less than 2 and theoretical plates of not less than 2000 determines the column efficiency. Likewise six replicates of standard solution were injected, the mean value calculated for each determination and % RSD should be less than 2%.

Specificity

If developed method have the ability of measuring analyte response even in the presence of potential impurities, the method is said to be specific. Also the absence of interferences of mobile phase and excipients with the principal analyte response peak denotes specificity. Sample and blank

solutions were prepared and analysed to check the interference of mobile phase with the analyte (CANAG).

Interference

Sample and blank solutions were prepared and analysed to check the interference of mobile phase with the analyte (CANAG).

Precision

Degree of scatter in terms of closeness of agreement among analyte responses from multiple sampling of the similar sample under prescribed conditions represents the precision. In the evaluation for precision, system precision, method precision and intermediate precision were carried out. Six replicates (100 μg/ml CANAG solution) of injections each for system and method precision was determined and examined. It was represented as % RSD of area counts of CANAG peak obtained. Ruggedness which is also represented as intermediate precision was carried out using different analysts, using different column, on different days and on different instruments makes. RSD for overall set of data was calculated.

Linearity

Standard stock solution was serially diluted to get the different concentration ranges from 20 μg/ml to 200 μg/ml of CANAG solution. At all concentration levels the peak responses were measured in triplicate. Linear regression analysis was carried out by plotting peak response versus concentration data. Linearity for CANAG response was determined by correlation coefficient (R^2) calculation, which shall not be less than 0.997.

Accuracy

Accuracy was performed using marketed formulation. Known amount of samples were taken in a clean dried volumetric flasks separately. To these flasks were spiked by the addition of known amount of CANAG standards in three different levels namely 60% (Level 1) 100% (Level 2) and 120% (Level 3) of sample concentration. The amount of CANAG recovered was calculated by subjecting these samples for analysis by developed method.

Robustness and Ruggedness

The capacity of an analytical method to remain unaffected by small but deliberate changes in method parameters measures robustness and by different instrument and analyst denotes ruggedness. The robustness was investigated by varying detection wavelength and flow rate. For each variation system suitability (theoretical plates and tailing factor) parameters were checked. For each set of data %RSD of area counts were determined. Overall %RSD between method precision data and each varied condition data represents the robustness. The developed method is said to be robust and rugged if conditions like system suitability should pass and overall %RSD not more than 2% were met.

LOD and LOQ

LOD represents lowest possible detectable analyte amount in a sample, not necessarily be quantified. LOQ represents the lowest amount of analyte for which quantification is possible by developed method with suitable precision and accuracy. The LOD and LOQ values of the developed method were obtained based on the standard deviation of the peak response and its slope as per ICH guidelines. This method involves the construction of the calibration curve from which slope and intercept were obtained. The standard deviation of the peak response was calculated by repeated injection of different concentration of drug solution to determine the standard deviation of intercepts of the regression line. Further, the LOD and LOQ of the developed method were obtained by the formula: LOD = 3.3 x (σ / S) and LOQ = 10 x (σ / S), where, σ = Standard deviation of the peak response of calibration curve and S = Slope of the calibration curve, respectively.

The percent relative standard deviation (%RSD) for each validation parameter were obtained by adopting descriptive statistics using MS Excel.

Estimation of Canagliflozin Hemihydrate in marketed product (Invokana)

Standard Solution: Standard stock solution was prepared by dissolving CANAG standard equivalent to 100 mg of Canagliflozin Hemihydrate in 100 ml of diluent. The above stock solution was diluted to get a final standard concentration of 100 μg/ml.

Sample Solution

Twenty tablets containing 100 mg of CANAG were weighed, average weight found and finely powdered. The sample solution was prepared by taking weight equivalent to 100 mg canagliflozin from powdered twenty tablets into 100 ml volumetric flask and kept for sonication with 75 ml methanol for 30 min with intermittent shaking. Allowed to cool to room temperature and made up the volume with methanol. 5 ml of this solution was diluted to 50 ml with mobile phase. Then the solution was filtered through 0.2 μm nylon membrane filter.

20 μl standard and sample solutions were injected to the system and chromatograms were recorded. The amount of CANAG was calculated. The calculations were performed on the comparative basis including the correction factor.

Forced degradation studies[11-14]

Forced degradation studies were performed to analyse the interference of degraded products peak on principle peak of drug. CANAG was subjected to stress conditions including acid and alkali hydrolysis, oxidative, thermal and photo degradation.

Stock solution preparation

Stock solution was prepared by accurately weighed 100 mg of CANAG standard, transferred in to a 100 ml volumetric flask, dissolved and diluted to 100 ml with diluent, to get a concentration of 1000 μg/ml.

Acid and alkali hydrolysis

10 ml of CANAG stock solution was transferred to a 100 ml volumetric flask. The solution was mixed with 10 ml of 0.1N HCl or 0.1N NaOH. The prepared solutions were kept at 80°C for 2 h in a water bath. The samples were cooled to room temperature. The solution was neutralized with an amount of acid or base equivalent to that of the previously added. Then volume was made up to 100 ml with the diluent to get a final concentration of 100 μg/ml.

Oxidative degradation

10 ml of CANAG stock solution was transferred to a 100 ml volumetric flask. The contents were then mixed with 10 ml of 3% hydrogen peroxide solution. The prepared solution was kept at 80°C for 2 h in water bath and the reaction mixture was allowed to proceed at room temperature with intermittent shaking. Then volume was made up to 100 ml with the diluent to get a final concentration of 100 μg/ml.

Thermal degradation

A standard drug of CANAG was exposed to a temperature of 80°C for 2 h in hot air oven. 100 mg of dried powder is weighed and transferred to a 100 ml volumetric flask, dissolved in diluent and the volume is made up to the mark with the same, to get a concentration of 1000 μg/ml, 1 ml of above stock solution was diluted to 10 ml with diluent to get a final concentration of 100 μg/ml.

Photolytic Degradation

10 ml of stock solution (1000 μg/ml) was exposed to UV light (254 nm) for 72 h. Then volume was made up to 100 ml with the diluent to get a final concentration of 100 μg/ml.
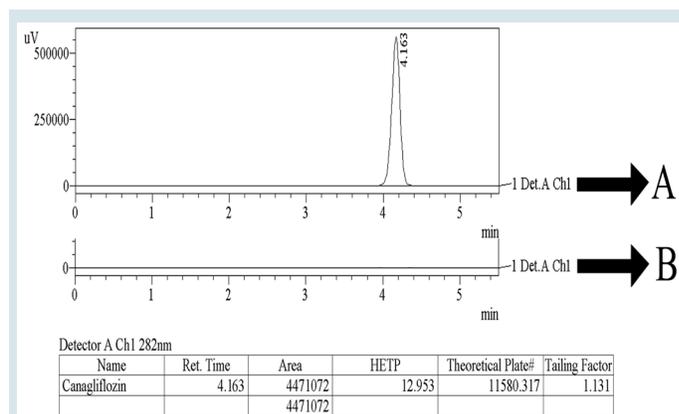
RESULTS AND DISCUSSION

HPLC method development

The novel chromatographic method for the quantification of CANAG was developed by varying chromatographic conditions detection wavelength, column, injection volume, mobile phase, pH of the mobile phase and runtime to achieve the shorter retention time. The method developed with 282 nm wavelength, COSMICSIL 100 C_{18} 5 μm column, injection volume of 20 μl, with water and acetonitrile in the ratio 30:70% v/v pH 3 adjusted with ortho-phosphoric acid, flow rate of 1.0 ml/min and for runtime of 6 min (Table 1). The mobile phase composition resulted in satisfactory separation and good symmetrical peak were observed with shorter retention time of 4.1 min. During the mobile phase preparation ortho phosphoric acid was used to adjust pH, thereby minimizing the human error in the preparation of buffer and mobile phase. Several pH were tried in neutral, alkali and acidic range, in acidic pH 3.0, symmetrical peak with shorter retention time was achieved. The injection volume chosen was 20 μl to achieve ideal theoretical plates. The developed method required no special type of stationary phase. Commonly used C_{18} column was employed. The chromatogram for CANAG and blank were depicted in Figure 2 suggested that there was no interference of mobile phase on drug peak at the achieved retention time.

Table 1: Chromatographic conditions for CANAG analysis.

Parameters	Conditions
Stationary Phase	C_{18} (250 × 4.6 mm), 5 μm column
Mobile Phase	Acetonitrile: Water (70:30%, v/v), adjusting the pH to 3.0±0.05 with ortho phosphoric acid
Flow rate (ml/min)	1.0
Run time (min)	6
Injection Volume (μl)	20
Detection Wavelength (nm)	282
Retention Time (min)	4.1

Name	Ret. Time	Area	HETP	Theoretical Plate#	Tailing Factor
Canagliflozin	4.163	4471072	12.953	11580.317	1.131
		4471072			

Figure 2: Chromatograms for CANAG (A) and Blank (B).

Method validation

System suitability

The newly developed analytical method before employing on analysis the system suitability has to be ensured. The system suitability parameters include retention time, theoretical plates and tailing factor for CANAG peak. These parameters were determined by 6 replicate injections and %RSD was calculated. The retention time was 4.13±0.02 min, theoretical plates obtained were 11580.32±0.43 with HETP value of 12.95±0.43 and tailing factor was 1.13±0.22. During the course of entire validation process these system suitability parameters were met.

Specificity

The chromatograms of drug and blank (mobile phase) were depicted in Figure 2 suggested that there are no co-eluting peaks from the blank and the presence of predominant sharp homogeneous peak of drug was observed. This indicates that the developed method has high specificity.

Precision

To establish precision of the newly developed analytical method for CANAG the quantification of drug in formulation were performed at intraday and interday and %RSD was calculated. The %RSD for all determinations were less than 2% in both intraday and interday suggested that the developed method was precise with respect to system and method. The values of quantification of drug in formulations at intraday and interday along with %RSD were depicted in Table 2.

Linearity

Linearity graph for pure CANAG plotted by obtaining area under the curve of respective chromatograms against different concentration as depicted in Figure 3. From the regression analysis the regression coefficient was found to 0.999 with slope and intercept of 38867.6 and 1642749 respectively. The R^2 value suggested that the linearity was achieved over the specified range (20-200 μg/ml).

Accuracy

To determine the accuracy of developed novel method of analysis a recovery studies by spiking known amount of standard at three levels into the sample solution and analysed. The results were ranged from 98.36±0.21 to 101.41±0.11% covering all the levels. The percentage recovery found to be within the range of 98-102% and %RSD less than 2 suggested that the developed method was accurate.

Range

The linearity range chosen was 20-200 μg/ml at this linearity range the developed method was accurate and precision was achieved.
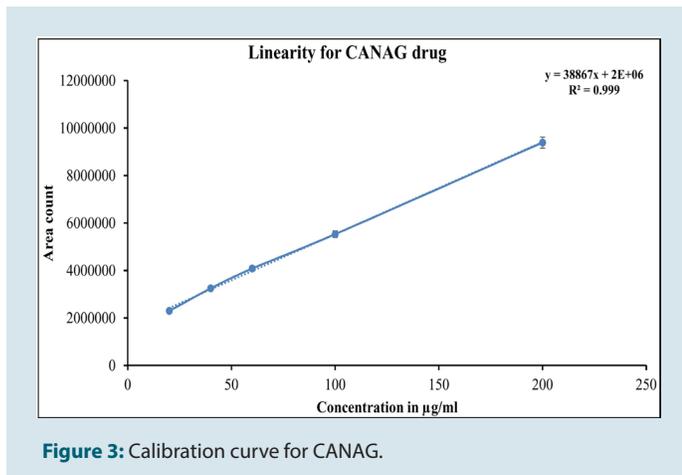
Figure 3: Calibration curve for CANAG.

Table 2: Results of CANAG method validation.

Canagliflozin hemihydrate concentration values found by validated method for various parameters (%)

Sl.No.	System	Method	Intraday H1	H2	H3	H4	H5	Interday Day 1	Day 2	Day 3	Change in Flow Rate 0.9mL	1.1mL	Change in Wave Length 279nm	283nm
1	100.585	99.926	101.946	101.485	102.309	102.935	104.029	100.455	101.467	103.383	99.775	99.798	99.947	99.789	100.002
2	100.321	99.989	101.241	101.808	103.256	102.740	104.428	100.130	100.718	103.330	99.814	99.973	99.923	99.924	100.117
3	100.518	99.841	101.302	101.613	103.430	102.840	103.806	99.901	101.499	103.372	99.908	99.997	99.957	100.049	99.898
4	100.552	98.763	101.783	101.876	103.379	102.842	104.237	99.777	101.817	103.405	100.317	100.053	100.068	100.032	100.081
5	100.669	100.001	101.058	101.520	103.188	102.907	104.121	99.981	101.178	103.403	100.435	100.228	100.062	100.104	99.951
6	100.529	100.216	101.875	101.660	103.113	102.984	104.324	100.510	101.051	103.392	99.750	99.952	100.043	100.101	99.951
STD DEV	0.11554	0.51791	0.37787	0.15561	0.41067	0.08610	0.22295	0.29957	0.38731	0.02781	0.29880	0.14048	0.06460	0.12215	0.08427
AVG	100.529	99.789	101.534	101.660	103.112	102.874	104.157	100.125	101.288	103.381	100.001	99.992	99.992	99.999	100.001
%RSD	0.11493	0.51901	0.37216	0.15307	0.39828	0.08370	0.21405	0.29919	0.38238	0.02690	0.29880	0.14048	0.06460	0.12215	0.08427

**Table 3: Estimation of CANAG in marketed product (Invocana).**

Brand Name	Standard Peak Area	Average	Sample Peak Area	Average	Amount Found/ Tablet (mg) ± %SD	Label Claim (mg)	% Amount Found
Invocana	4451937 4543377 4549096	4514803.3	5094026 5003341 4998804	5032057	103.37±1.07	100	100.4

*n=3

Table 4: Results of CANAG forced degradation studies.

Sl. No.	Nature of Stress	Retention Time (min) ±SD*	Area ±SD*	% Degraded
1	Acid hydrolysis	4.162±0.866	3965922±0.568	5.32
2	Base hydrolysis	4.164±0.004	4073585±0.403	2.75
3	Oxidation at 80°C	4.178±0.036	3993772±0.549	4.65
4	Thermal degradation at 80°C	4.157±0.011	4056922±0.449	3.14
5	Photo degradation UV 254 nm	4.156±0.001	4171926±0.169	0.39

*n=3
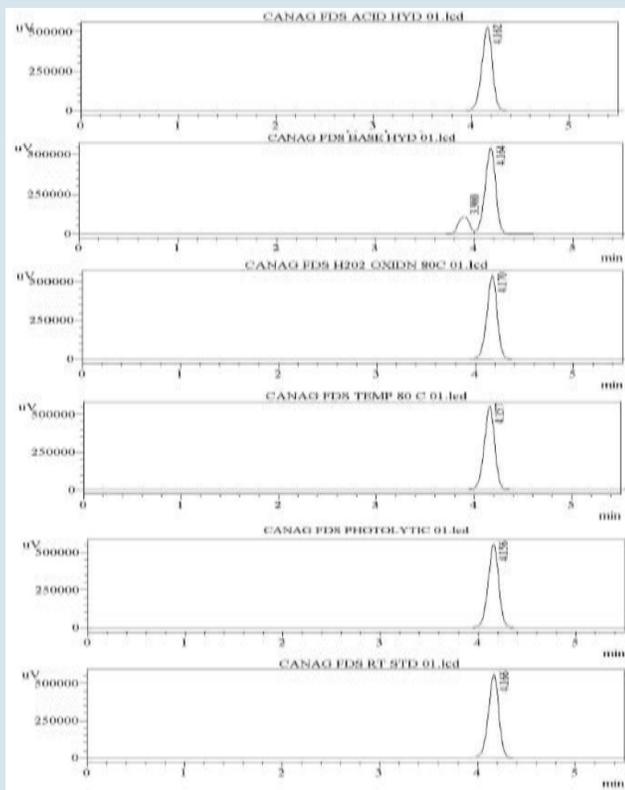
Figure 4: Forced degradation study of CANAG; HPLC chromatographs for different stressed Conditions.

Robustness and Ruggedness

To confirm robustness of the developed analytical method, deliberately varied the chromatographic conditions such as detection wavelength (±2 nm) and flow rate (±0.1 ml). For ruggedness the analysis was performed using different instrument and analyst. The quantification of drug was carried out and the %RSD were within the limit of less than 2% and depicted in Table 2. Suggested that the developed method to be robust and rugged.

LOD and LOQ

In this method LOD was found to be 0.28 μg/ml and LOQ was found to be 0.78 μg/ml.

Quantification of CANAG in tablet dosage form

The newly developed and validated method was employed to quantify CANAG in marketed tablet dosage form (Invocana). The method involved was preparation of three different sample solution from each sample solution and obtained the chromatogram in triplicate. The average assay value of each trials with %RSD was 103.37±1.07% (Table 3). The assay values obtained were within the acceptance limits (90-110%) against the amount claimed on the label.

Forced Degradation Studies

The results of different stress conditions of forced degradation studies of CANAG were as follows. In alkali stress the degradation was 2.75%, in acid stress condition 3.32%, in oxidative stress 4.65%, in thermal and photolytic stress the degradation were 3.14% and 0.37% respectively (Table 4). Relatively the rate of degradation was more in acid stress condition whereas the degradation is negligible in photolytic stress. Among all the chromatograms of forced degradation studies, a degradation peak of minimal intensity was observed at 3.9 min retention time in alkali stress condition. The respective chromatograms at different stress conditions are illustrated in Figure 4. As the principle peaks obtained after forced degradation studies were homogeneous, symmetrical and not influenced by the presence of degradation products. This suggested that the newly developed and validated analytical method for CANAG is stability indicating in nature. Thereby this method can be employed to determine CANAG in presence of its degradation products.

CONCLUSION

The present research study concluded that the newly developed and validated method of analysis for CANAG was simple, rapid and economical. During the course of validation, the developed method was found to be specific, precise, accurate, robust and rugged. The forced degradation study attributed that the analysis of CANAG was not interfered by the presence of its degraded products at different stress conditions. Hence the developed method was stability indicating in nature. The newly

developed and validated method can be adopted for the quantification of CANAG in bulk drug and as API in formulation.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

Authors declare no conflict of interest.

## ABBREVIATIONS

RP-HPLC: Reverse Phase High Performance Liquid Chromatograpy; **CANAG:** Canagliflozin; **API:** Active Pharmaceutical Ingredient; **ICH:** International Conference on Haromonization; **RSD:** Relative Standard Deviation; **HPTLC:** High Performance Thin Layer Chromatography; **UV:** Ultra-Violet; **LOD:** Limit of Detection; **LOQ:** Limit of Quantfication, **FTIR:** Fourier-transform Infra-red; **MS:** Microsoft; **HETP:** Height Equivalent to the Theoretical Plates.

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PICTORIAL ABSTRACT
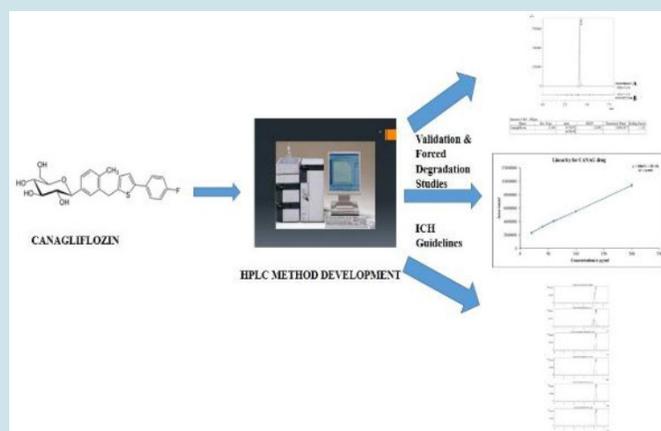
SUMMARY

- In the present research work the attempt was made to develop and validate a novel RP-HPLC method with stability indicating property for CANAG and same method adopted to quantify CANAG as bulk drug and API in formulation.

- As the requirement of ICH guidelines, the developed novel analytical method was validated. The drug CANAG was subjected to forced degradation to analyse the interference of degraded products on principle peak of CANAG. The chromatographic conditions of developed method were 282 nm wavelength, COSMICSIL 100 C_{18} 5 µm column, injection volume of 20 µl, with water and acetonitrile in the ratio 30:70% v/v pH 3 adjusted with ortho-phosphoric acid, flow rate of 1.0 ml/min and for runtime of 6 min.

- The present research study concluded that the newly developed and validated method of analysis for CANAG was simple, rapid and economical. The forced degradation study attributed that the analysis of CANAG was not interfered by the presence of its degraded products at different stress conditions. Hence the developed method was stability indicating in nature. The newly developed and validated method can be adopted for the quantification of CANAG in bulk drug and as API in formulation.