INTERNATIONAL JOURNAL OF MEDICAL, PHARMACEUTICAL AND BIOLOGICAL SCIENCES
(eISSN : 2832-787X, pISSN : 2832-7888)
Journal Home Page: http://www.aphinfo.com/ijmpbs

Method development and validation of a RP-HPLC method for the simultaneous estimation of Metformin and Canagliflozin in the presence of their degradation product

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ABSTRACT

The current study outlines the development and validation of a robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of metformin and canagliflozin in the presence of their degradation products. Using an optimized Hypersil C18 column and an isocratic mobile phase, this method offers high sensitivity, precision, and specificity, essential for effective analysis of pharmaceutical formulations. Comprehensive validation under ICH guidelines was performed to assess parameters including linearity, precision, accuracy, robustness, and stability.

Keywords: RP-HPLC, Metformin, Canagliflozin, Method validation, Degradation studies, Simultaneous estimation.

Received: 5 July 2024 / Revised: 28 July 2024 / Accepted: 6 November 2024 / Available online: 31 December 2024

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INTRODUCTION

Metformin and canagliflozin are frequently co-administered for the management of type 2 diabetes, offering complementary effects on glucose regulation. However, accurately quantifying both drugs in a single analysis presents challenges due to their distinct physicochemical properties and potential for degradation. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a preferred technique for simultaneous drug quantification due to its precision, adaptability, and ability to separate compounds effectively [1, 2].

RP-HPLC methods for metformin and canagliflozin have previously focused on individual drug analysis or dual quantification without stability studies [3, 4]. This study aims to fill this gap by developing a single, validated RP-HPLC method capable of simultaneous estimation of metformin and canagliflozin, while also identifying their degradation products [5-7]. Utilizing an optimized Hypersil C18 column and isocratic mobile phase, this method is tailored for high selectivity and sensitivity, which are crucial for accurate measurement in the presence of degradation products [8, 9].

Degradation studies are fundamental in pharmaceutical analysis, as they assess drug stability under various stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments [10,11]. These forced degradation tests, recommended by the International Council for Harmonisation (ICH), ensure that the developed method is not only precise but also capable of distinguishing active ingredients from degradation by-products [12,13].

The study validates the method following ICH guidelines for system suitability, linearity, accuracy, precision, robustness, and limits of detection (LOD) and quantitation (LOQ) [14]. Additionally, the method is tested on commercially available formulations to confirm its efficacy in real-world applications [15].

MATERIALS AND METHODS

Instrumentation

Analytes were scanned between 200-400 nm using UV-visible spectrophotometer (model UV- 1700, Shimadzu). Experiments were carried out using Shimadzu Prominence Modular HPLC system with LC 20AT solvent delivery unit, CBM 20A system controller,

SIL 20A auto- sampler, CTO 20A column oven and SPD 20 A UV Detector. Spinchrom software was used as the data integrator. 20µL fixed-loop injector was used for the injection of the samples with the flow rate of 1.0 mL min^{-1}. The pH of the solutions was measured with the pH meter (S20K, Mettler Toledo). The drugs in specific degradation conditions were refluxed using a rotavapor (R-300, Buchi). A high precision analytical balance (ATX-124, Shimadzu) was used for weighing.

Reagents and Chemicals

The reference materials of metformin and canagliflozin were purchased from Mesochem Technology, Inc., Beijing, China. Methanol and water of HPLC grade were used and purchased from Fisher Scientific, Hyderabad, India. Potassium dihydrogen phosphate was purchased from Sigma-Aldrich Company, Bangalore, India. Commercial tablets (Invokamet: dosage metformin 500 mg and canagliflozin 150 mg) were purchased froma local pharmacy.

Selection of Wavelength

Standard solution of metformin (10 µg/mL) and canagliflozin (10 µg/mL) were scanned between 200-400 nm using a UV-visible spectrophotometer. The wavelength overlay spectra ofabove solutions was used to select the specific wavelength.

Chromatographic Separation

Analytes were separated on Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (50:50 v/v). The detection was carried out at the wavelength of 225 nm. Peak area, peak height, retention time and resolution were recorded using Spinchrom software. 20µL fixed-loop injector was used for the injection of the samples with the flowrate of 1.0 mL min^{-1}.

Preparation of Standard Solutions

Metformin (10 mg) and canagliflozin (10 mg) were weighed separately. Final concentration of individual standard stock solutions of metformin (100 µg/mL) and canagliflozin (100 µg/mL) were made with methanol and stored at 4°C refrigerator untilused. Working stock solution containing metformin (10 µg/mL) and canagliflozin (10 µg/mL) was prepared with mobile phase.

Method Validation

System Suitability Test

System suitability test is an integral part of the chromatographic method. These tests areused to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use [40].

Linearity

Linearity was assessed by analysis of combined standard solution in a range of 5- 20 µg/mL for each of the metformin and canagliflozin.

Precision

Results were expressed as percent relative standard deviation (%RSD) or coefficient of variance.

Repeatability

A standard solution containing 10 µg/mL of each of the metformin and canagliflozin was injected six times, areas of peaks were measured and % RSD was calculated to determine the repeatability of the method.

Intra- Day and Inter-Day Precision

A standard solution containing 5, 10 and 15 µg/mL of each of the metformin and canagliflozin were analyzed three times on the same day for the determination of intra- day precision and on three different days for the determination of inter-day precision and % RSD was calculated.

Accuracy

Accuracy was calculated at three different levels in terms of % recovery by spiking known amount of standard solution (80%, 100% and 120%) to the

solution of a synthetic mixture of marketed formulations.

Specificity and Selectivity

The specificity of the method was established through the study of resolution factors of the drug peak from the nearest resolving peak and also among all other peaks.

Limit of Detection and Limit of Quantitation (LOD and LOQ)

The LODs and LOQs were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Robustness

Robustness of the method was investigated by varying the chromatographic conditions, such as, changing the flow rate by ± 10% (i.e. 0.8 mL/min and 1.2 mL/min), changing the ratio of mobile phase was with ±2 (i.e. buffer: methanol (48:52) and buffer: methanol (52:48)), and changing the pH of the buffer in the mobile phase with ± 0.2% (i.e. 2.8 and 3.2). Robustness of the developed method was indicated by the overall % RSD between the data, at each variable condition.

Analysis of Marketed Formulation

Twenty tablets of marketed formulation Invokamet with the label claim of 500 mg of metformin and 150 mg of canagliflozin were weighed individually and finely powdered. Tablet powder equivalent to 50 mg of metformin and 15 mg canagliflozin was weighed accurately and transferred to a 100 ml volumetric flask. The analytes were extracted with 5 ml methanol by sonication in the ultra-sonicator bath and then the volume was made up to the mark with mobile phase to obtain the concentration of 20 µg/mL for metformin and 6 µg/mL for canagliflozin. Samples were analyzed using the developed assay. The areas of resulting peak were measured at 225 nm.

Stress Degradation Studies

Acid hydrolysis

Forced degradation in acidic condition was performed by adding 1 mL of standard solution of mixtures of metformin (100 µg/mL**)** and canagliflozin (100 µg/mL) to 6 mL each of methanol: water (1:1) To start the reaction, pH 3.0 was adjusted with 0.1 M hydrochloric acid and refluxing the mixture at 70°C for 2 hours. The solution was then allowed to reach at room temperature, neutralized to pH 7 by the addition of 0.1 M sodium hydroxide. The final concentration was made with mobile phase to get 10 µg/mL for each of metformin and canagliflozin, respectively. Tablet powder equivalent to 50 mg of metformin and 15 mg canagliflozin was also treated with described acidic conditions.

Alkaline hydrolysis

Alkali-induced, forced degradation was performed by adding 1 mL of a standard solution of a mixture of metformin (100 µg/mL) and canagliflozin (100 µg/mL) to 6 mL methanol: water (1:1). Adjusting the pH 12.0 with 0.1 M sodium hydroxide and refluxing the mixture at 70°C for 2 hours started the alkaline hydrolysis. The solution was then allowed to reach at room temperature and neutralized to pH 7.0 by the addition of 0.1 M hydrochloric acid. The mixture was diluted with mobile phase to get a final concentration of 10 µg/mL for each of metformin and canagliflozin, respectively. Tablet powder equivalent to 50 mg of metformin and 15 mg canagliflozin was also treated with described alkaline conditions.

Oxidative degradation

To evaluate the effect of oxidizing conditions, 1 mL of the standard solution of a mixture of metformin (100 µg/mL) and canagliflozin (100 µg/mL) was added to 2 mL of 3% hydrogen peroxide solution and the mixture was refluxed at 70°C for 2 hours. The solution was then allowed to reach room temperature and diluted to 100 ml with the mobile phase to get a final concentration of 10 µg/ml for each of Metformin and Canagliflozin respectively. Tablet powder equivalent to 50 mg of Metformin and 15 mg Canagliflozin was also treated with described oxidative degradation conditions.

Thermal degradation

To evaluate the effect of temperature, 1 ml of a standard solution of a mixture of Metformin (1

mg/mL) and Canagliflozin (1 mg/mL) was stored at 105°C in a hot air oven for 1.5 hours. The solution was then allowed to reach room temperature and diluted to 100 ml with the mobile phase to get a final concentration of 10 µg/ml for each of Metformin and Canagliflozin respectively. Tablet powder equivalent to 50 mg of Metformin and 15 mg Canagliflozin was also treated with described thermal degradation condition.

Photolytic degradation

To study the effect of UV light, a mixture of Metformin and Canagliflozin, 25 mg each, was exposed to short and long wavelength UV light (254 nm and 366 nm, respectively) for 24 hours, and then dissolved in 10 ml of methanol. The volume was made up by the mobile phase in a 50 ml volumetric flask and then 1 ml of stock solution was further diluted with the mobile phase to give a solution of final concentration equivalent to 10 µg/ml for each of Metformin and Canagliflozin. Tablet powder equivalent to 50 mg of Metformin and 15 mg Canagliflozin was also treated with described photolytic degradation conditions.

Twenty microliters of the resulting solutions were injected into the HPLC system and the chromatograms were recorded.

RESULTS AND DISCUSSION

Method Development

As Metformin and Canagliflozin both showed absorbance response at a wavelength of 225 nm, it was selected as a wavelength of detection. Fig. 1 represents the overlying UV spectra of Metformin and Canagliflozin.

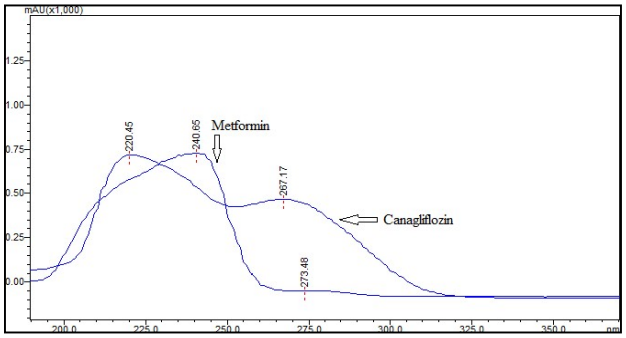

Figure 1: Overlay UV Spectrum of Metformin and Canagliflozin showing selection of wavelength Detection

For method development, reverse phase liquid chromatography was chosen because of its simple and convenient use in terms of efficiency, stability and reproducibility. Analytes were separated on Hypersil C18, 250 x 4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (50:50 v/v). Analytes were detected at 225 nm. 20µL fixed-loop Method Development and Validation of a Reversed-Phase Liquid Chromatographic Method (RP-HPLC) for the Simultaneous Estimation of Metformin and Canagliflozin in the Presence of Their Degradation Products injector was used for the injection of the samples with the flow rate of 1.0 mL min−1. Retention time was 3.47 min and 5.283 min for Metformin and Canagliflozin, respectively, as shown in Fig. 2.

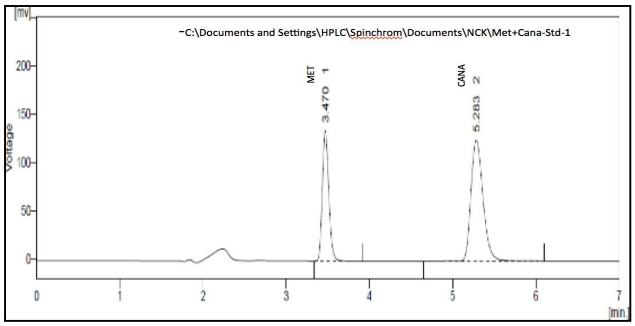

Figure 2: Chromatogram of Metformin and Canagliflozin in 50 Mm Potassium Di-Hydrogen Phosphate Buffer (Ph 3.0): Methanol (50:50 V/V) with Flow Rate-1.0Ml/Min

Method Validation

The method was validated as per ICH guidelines with respect to parameters like linearity, precision, accuracy, specificity, and robustness.

The number of theoretical plates, peak tailing and resolution factor were determined to define system suitability parameters for Metformin and Canagliflozin. The results for system suitability data are listed in Table 1.

Table 1: System Suitability parameters For Metformin and Canagliflozin

System Suitability Parameters	Metformin	Canagliflozin
Theoretical plates per column (N)	9605	6300
Symmetry factor/Tailing factor	1.286	1.417
Resolution	8.892	

Linearity and range were assessed by analysis of combined standard solution in the range of 5-20 µg/ml for Metformin and Canagliflozin, each respectively. Standard calibration curve for Metformin and Canagliflozin are represented as Fig. 3 and 4, respectively.

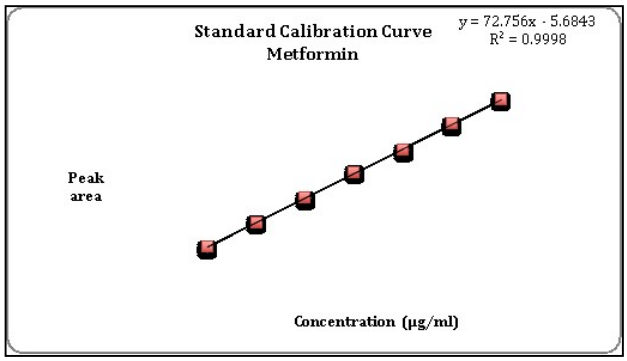

Figure 3: Standard Calibration Curve of Metformin (5-20 Mg/Ml)

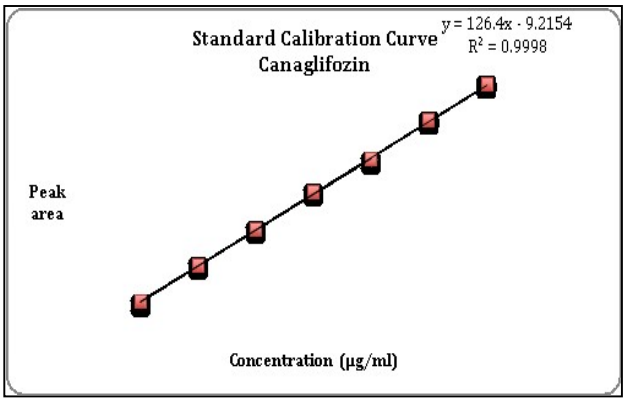

Figure 4: Standard Calibration Curve of Canagliflozin (5-20 Mg/Ml)

The data for regression analysis is listed in Table 2. A standard solution containing 10 µg/ml for each of Metformin and Canagliflozin respectively was injected six times and areas of peaks were measured to determine the repeatability of the method. % R.S.D. value for the determination of repeatability is represented in Table 3.

Table 2: Results from Regression Analysis for Metformin and Canagliflozin

Description	Metformin	Canagliflozin
Linearity and range	5-20 µg/ml	5-20 µg/ml
Regression co-efficient	0.999	0.999
Slope (m)	72.75	126.4
Intercept (c)	5.684	9.215

Table 3: Repeatability Data for Metformin and Canagliflozin

Metformin				Canagliflozin			
Conc. (µg/ml)	Peak Area	Mean ± S.D (n=6)	% R.S.D	Conc. (µg/ml)	Peak Area	Mean ± S.D (n=6)	% R.S.D
10	722.142	724.045±3.522	0.486	10	1258.405	1253.955 ±6.251	0.499
	719.964				1248.954		
	723.561				1253.564		
	722.103				1245.595		
	727.161				1254.289		
	729.340				1262.922		

A standard solution containing (5,10,15 µg/ml) Metformin and (5,10,15 µg/ml) Canagliflozin were analyzed three times on the same day for the determination of intra-day precision and on three different days for the determination of inter-day precision. % R.S.D values for intra- day and inter-day precision are represented in Table 4. The accuracy of the method was confirmed by recovery study from the synthetic mixture of marketed formulation at three levels of standard addition. The results are shown in Table 5.

Table 4: Intra-day and Inter-Day Precision for Metformin and Canagliflozin

Metformin			Canagliflozin		
Conc. (µg/ml)	Mean ± S.D (n=6)	% R.S.D	Conc. (µg/ml)	Mean ± S.D (n=6)	% R.S.D
Intra-day precision					
5	359.241±0.367	0.102	5	625.084±3.405	0.545
10	729.132±2.773	0.380	10	1261.812±2.860	0.226
15	1087.211±2.433	0.224	15	1885.129±9.016	0.478
Inter-day precision					
5	359.046±0.667	0.186	5	621.383±4.539	0.730
10	724.270±6.917	0.955	10	1244.327±15.397	1.237
15	1073.888±10.787	1.004	15	1856.234±28.808	1.552

Table 5: Accuracy in Terms of % Recovery for Metformin and Canagliflozin

Conc. Level (%)	Sample amount (µg/ml)	Amount of Standard Added (µg/ml)	Metformin			Canagliflozin		
			Amount Recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D	Amount Recovered (µg/ml)	% Recovery	% Mean Recovery ±S.D
80%	5	4	4.038	100.949		3.978	99.443	99.961± 1.187
	5	4	4.029	100.722	100.994 ± 0.296	3.965	99.122	
	5	4	4.052	101.310		4.053	101.319	
100 %	5	5	5.025	100.498		5.004	100.074	98.988± 0.942
	5	5	5.025	100.493	100.304 ± 0.332	4.925	98.492	
	5	5	4.996	99.921		4.920	98.397	
120 %	5	6	6.027	100.447		6.052	100.872	100.002 ± 1.652
	5	6	6.008	100.132	100.399 ± 0.247	5.886	98.097	
	5	6	6.037	100.619		6.062	101.037	

Percentage recovery was in the range of 100.304 - 100.994% for Metformin and 98.988 -100.002 % for Canagliflozin. LOD was 0.358 µg/ml and 0.335 µg/ml for Metformin and Canagliflozin, respectively, whereas the LOQ was 1.085 µg/ml and 1.015 µg/ml for Metformin and Canagliflozin respectively. Method was robust with % RSD values <2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in the flow rate. Applicability of the proposed method was evaluated by analyzing a synthetic mixture of marketed formulation (Invokamet, n=6) and the results are shown in Table 6. The assay results were 98.926 % and 98.526 % to labeled value of Metformin and Canagliflozin respectively in marketed tablet dosage form.

Table 6: Analysis of Marketed Formulation by developed method

INVOKAMET Tablet	Synthetic mixture	
mg/tablet powder	Metformin (500 mg)	Canagliflozin (50 mg)
Assay (% of label claim*) Mean ± S. D.(n=6)	98.926±0.151	98.526±0.441
% RSD	0.57	0.69

Establishment of Stability Indicating Method for Assessment of Degradation Behavior

The stressed samples were assayed using developed RP-HPLC method. Following degradation behavior was observed under different stress conditions for the high-performance liquid chromatography studies on the combination of Metformin and Canagliflozin [Table 7-8].

Table 7: Percent Degradation of Metformin with Retention Time of the DegradationProducts

Sr. No.	Conditions	Retention timeof Metformin / degradation products (min)	Average Peakarea of Metformin Standard	% degradation of Metformin (n=5)	Average Peakarea of Metformin intablet (n=5)	% degradation ofMetformin in tablet (n=5)
1	Untreated stock solution (10µg/ml)	3.47	779.798	-	-	-
2	Acid hydrolysis	4.01, 4.19	680.561	12.726	646.374	17.110
3	Alkali hydrolysis	4.15, 4.33	601.732	22.835	602.454	22.742
4	Oxidative degradation	3.97,4.43,4.94	669.313	14.168	654.393	16.082
5	Thermal degradation	3.91,4.06,4.58	568.767	27.062	557.754	28.475
6	Photolytic degradation	4.13,4.36,4.62	619.601	20.543	602.377	22.752

Table 8: Percent Degradation of Canagliflozin with Retention Time of the Degradation Products

Sr. No.	Conditions	Retention time of Canagliflozin / degradation products (min)	Average Peakarea of Canagliflozin Standard	% degradation of Canagliflozin(n=5)	Average Peakarea of Canagliflozin in tablet (n=5)	% degradation of Canagliflozin in tablet (n=5)
1	Untreated stock solution (10µg/ml)	5.283	1007.218	-	-	-
2	Acid hydrolysis	5.98, 6.29, 6.72, 7.29	817.280	18.858	793.604	21.208
3	Alkali hydrolysis	5,75, 6.28	840.392	16.563	811.224	19.459
4	Oxidative degradation	6.86, 7.42, 8.38, 9.18	844.669	16.138	811.160	19.465
5	Thermal degradation	3.91, 4.06, 4.58, 6.92	764.701	24.078	746.485	25.886
6	Photolytic degradation	4.13, 4.36, 4.62, 6.32	738.665	26.663	768.675	23.683

Significant degradation was observed in the presence of acidic, basic, neutral oxidative and photolytic stress conditions for Metformin and Canagliflozin, respectively (n=5). Percentage Degradation for the standard drug was 13%, 23%, 14%, 27% and 20%for Metformin and 18%, 16%, 16%, 24% and 26% for Canagliflozin in the presence of acidic, basic, thermal, oxidative and photolytic degradation, respectively. Percentage Degradation for the Metformin in INVOKAMET tablet was 17%, 22%, 16%, 28% and 22% in the presence of acidic, basic, thermal, oxidative and photolytic degradation, respectively. Percentage degradation for Canagliflozin in INVOKAMET was 21%, 19%, 19%, 25% and 23% in the presence of acidic, basic, thermal, oxidative and photolytic degradation, respectively. The percent degradation was calculated by the formula: % degradation = (Average peak area of untreated stock solution – average peak area of stock solution under specific degradation condition)/(average peak area of untreated stock solution) x 100)

CONCLUSION

Proposed reversed phase high performance liquid chromatographic method was able to successfully separate, identify and quantify Metformin and Canagliflozin simultaneously in the presence of their degradation products. This implies the stability indicating nature and specificity of the method. The

developed validated stability indicating RP-HPLC method is simple, precise, accurate, robust and reproducible resolving all the degradation products from the analytes of interest. Thus, the proposed method can be applied for the determination of Metformin and Canagliflozin in bulk drug, pharmaceutical pre-formulation and formulations development studies in pharmaceutical research laboratories.

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