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A Novel Validated RP-HPLC-DAD Method for the Simultaneous Estimation of Metformin Hydrochloride and Canagliflozin in Bulk and Pharmaceutical Tablet Dosage form with Forced Degradation Studies

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ABSTRACT

A novel approach was used to develop and validate a rapid isocratic Reversed Phase-High Performance Liquid Chromatographic method for the simultaneous estimation of Metformin Hydrochloride and Canagliflozin in bulk and pharmaceutical tablet dosage form with forced degradation studies. The separation was performed by using Kromasil C_{18} column (250mm×4.6 mm, 5mm particle size), Waters Alliance e2695 HPLC system with 2998 PDA detector and mobile phase contained a mixture of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v). The flow rate was set to 1ml/min with responses measured at 254nm. The retention time of Metformin Hydrochloride and Canagliflozin was 2.440min and 3.713min respectively with resolution of 8.95.Linearity was established in the range of 50-300µg/ml for Metformin Hydrochloride and 5-30µg/ml for Canagliflozin with correlation coefficients (r^2=0.999). The percentage recoveries were between (99.45%-100.65%) and (99.95%-100.74%) for Metformin Hydrochloride and Canagliflozin respectively. Validation parameters were evaluated according to the International Conference on Harmonization (ICH) Q2 R1 guidelines. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation and water. Metformin Hydrochloride and Canagliflozin are more sensitive towards oxidative degradation condition. The developed method was successfully applied for the quantification and hyphenated instrumental analysis.

Key words: Metformin Hydrochloride, Canagliflozin, PDA detector, Hyphenated, ICH.

INTRODUCTION

Metformin Hydrochloride

Metformin Hydrochloride is an orally administered biguanide derivative used to lower blood glucose concentrations in patients with non-insulin-dependent diabetes mellitus[1]. Metformin Hydrochloride improves insulin sensitivity and decreases insulin resistance by inhibiting Complex 1 of the mitochondrial respiratory chain and inducing

AMP-activated protein kinase-dependent signaling[2, 3]. Metformin Hydrochloride is chemically known as 1, 1-Dimethylbiguanide monohydrochloride were shown in (Figure 1).

Canagliflozin

Canagliflozin is an antidiabetic drug used to improve glycemic control in patients with type 2 diabetes. Canagliflozin is an inhibitor of subtype 2 sodium-glucose transport protein (SGLT2), which is responsible for at least 90% of the glucose reabsorption in the kidney (SGLT1 being responsible for the remaining 10%)[4]. Canagliflozin is chemically known as (2*S*, 3R, 4R, 5S, 6R)-2-{3-[5-[4-Fluoro-phenyl)-thiophen-2-ylmethyl]-4-methyl-phenyl}-6-hydroxymethyl-tetrahydro-pyran-3, 4, 5-triol was shown in (Figure 2). Literature review reveals that very few analytical methods have been reported for the determination of Metformin Hydrochloride and Canagliflozin which include ultra performance liquid chromatography[5], high performance liquid chromatography with UV detection[6,7], Spectrophotometric method[8-13],UHPLC-MS/MS[14], Solid Phase Extraction-Non-aqueous capillary electrophoresis [15] and bioequivalence studies[16]. The present study was aimed to develop a novel, simple, accurate, precise, economic and validated method for the simultaneous estimation of Metformin Hydrochloride and Canagliflozin with forced degradation studies according to ICH guidelines [17].

EXPERIMENTAL

Chemicals and reagents

Metformin Hydrochloride (API) was obtained from Rantus Pharma Pvt. Ltd., Hyderabad, India and Canagliflozin (API) was obtained from Manus Aktteva Biopharma Ltd., Ahmedabad, Gujarat. HPLC grade of Ammonium Acetate was obtained from Rankem Ltd., India and HPLC grade of Acetonitrile was obtained from Merck Specialities Private Limited, India. HPLC grade of Water and Ortho phosphoric acid was obtained from Rankem Ltd., India. Invokamet tablet contains Metformin Hydrochloride 500mg and Canagliflozin 50mg were kindly supplied by Janssen Pharmaceuticals, Inc.

Instrumentation

The analysis was performed by using a chromatographic system from Waters Alliance e2695 HPLC system with 2998 PDA detector. The HPLC system was equipped with Empower 2 software. Semi-micro analytical balance (India), Ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), Digital pH meter (Systronics model 802) and Whatmann filter paper No. 41 (Whatmann International Ltd., England) were used in the study.

Chromatographic conditions

Metformin Hydrochloride and Canagliflozin was analysed in Kromasil C_{18} column (250mm×4.6 mm, 5mm particle size) column for the chromatographic separation. The mobile phase was composed of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v). Filtered through 0.45µm nylon membrane filter under vacuum filtration and pumped at ambient temperature, at a flow rate of 1 ml/min with UV detection wavelength at 254nm. Injection volume was 20ìl. The run time was 8 min and the retention time of Metformin Hydrochloride and Canagliflozin was found to be 2.440min and 3.713min respectively with resolution of 8.95. The resulting HPLC chromatogram was shown in (Figure 6).

Chromatographic Parameters

Equipment	:	Waters Alliance e2695 HPLC system with 2998 PDA detector
Column	:	Kromasil C_{18} column (250 mm ×4.6 mm, 5mm particle size)
Flow rate	:	1ml/min
Wavelength	:	254 nm
Injection volume	:	20 ml
Column oven	:	Ambient
Run time	:	8 Minutes

Solutions and sample preparation

Preparation of Phosphate buffer

A 0.01M Ammonium acetate buffer was prepared by dissolving 0.77gm of Ammonium acetate in 1000ml of HPLC grade water and pH was adjusted to 3.5 with orthophosphoric acid. The buffer was filtered through 0.45ìm nylon membrane filter to remove all fine particles and gases.

Preparation of mobile phase

The above prepared Ammonium acetate

buffer and Acetonitrile HPLC grade were mixed in the proportion of 65:35, v/v and was filtered through 0.45µm nylon membrane filter and degassed by sonication.

Preparation of diluent

Mobile phase was used as diluent.

Preparation of standard stock solutions of Metformin Hydrochloride and Canagliflozin

Standard stock solutions of Metformin Hydrochloride and Canagliflozin were prepared by dissolving 500mg of Metformin Hydrochloride and 50mg of Canagliflozin in100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 ìm nylon membrane filter and degassed by sonicator to get the concentration of 5000µg/ml of Metformin Hydrochloride and 500µg/ml of Canagliflozin.

Preparation of standard solutions of Metformin Hydrochloride and Canagliflozin for assay

From the above standard stock solution of 5000µg/ml of Metformin Hydrochloride and 500µg/ml of Canagliflozin further pipette 0.4ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin.

Preparation of sample solutions of Metformin Hydrochloride and Canagliflozin

Twenty tablets were accurately weighed and powdered and tablet powder equivalent to 500mg of Metformin Hydrochloride and 50mg of Canagliflozin were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent. Further pipette out 0.4ml from the above Metformin Hydrochloride and Canagliflozin sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin. 20ml from standard and sample solution were injected into the chromatographic system and the peak areas were measured for Metformin Hydrochloride and Canagliflozin which was shown in (Figure 6 and 7) and the % assay was calculated by comparing the peak area of standard and sample chromatogram by using the formula given below and the assay results was shown in (Table 1).

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = Average peak area of sample preparation
AS= Average peak area of standard preparation
WS = Weight of standard taken in mg
WT=Weight of sample taken in mg
P = Percentage purity of working standard
DS= Dilution factor for standard preparation
DT=Dilution factor for sample preparation

Selection of wavelength

In simultaneous estimation of Metformin Hydrochloride and Canagliflozin isosbestic wavelength is used. Standard stock solutions of Metformin Hydrochloride and Canagliflozin were prepared by dissolving 500mg of Metformin Hydrochloride and 50mg of Canagliflozin in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45ìm nylon membrane filter and degassed by sonicator to get the concentration of 5000µg/ml of Metformin Hydrochloride and 500µg/ml of Canagliflozin. From the above standard stock solution of 5000µg/ml of Metformin Hydrochloride and 500µg/ml of Canagliflozin further pipette 0.4ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin. The wavelength of maximum absorption (ëmax) of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The isosbestic wavelength (ëmax) was found to be 254nm for the combination shown in (Figure 3).

Method validation

The developed method for the simultaneous estimation of Metformin Hydrochloride and Canagliflozin was validated as per the ICH guidelines for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitation (LOQ) [17].

System suitability

At first the HPLC system was optimized as per the chromatographic conditions. One blank followed by six replicates of a single calibration standard solution of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin was injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, peak asymmetry and resolution were taken and results were presented in (Table 2).

Specificity

The effect of excipients and other additives usually present in the combined tablet dosage form of Metformin Hydrochloride and Canagliflozin in the determination under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the blank and placebo solution into the HPLC system. The representative chromatogram of blank and placebo was shown in (Figure 4 and 5).

Linearity and range for Metformin Hydrochloride and Canagliflozin

Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6ml of mixed standard working solutions of Metformin Hydrochloride and Canagliflozin was pipette out from the standard stock solution of 5000µg/ml of Metformin Hydrochloride and 500µg/ml of Canagliflozin and transferred into a series of 10ml clean dry volumetric flask and make volume up to the mark with the same diluent to get the concentration of 50, 100, 150, 200, 250 and 300µg/ml of Metformin Hydrochloride and 5, 10, 15, 20, 25 and 30µg/ml of Canagliflozin. The calibration standard solutions of Metformin Hydrochloride and Canagliflozin were injected using a 20ìl Hamilton Rheodyne injector and the chromatograms were recorded at 254nm and a calibration graph was obtained by plotting peak area versus concentration of Metformin Hydrochloride and Canagliflozin respectively. The linearity data is presented in (Figure 26) and (Table 3).

Acceptance Criteria: Correlation coefficient should be not less than 0.999

Accuracy studies for Metformin Hydrochloride and Canagliflozin

The accuracy of the method was determined by calculating recovery of Metformin Hydrochloride and Canagliflozin by the method of standard addition. Known amount of standard solution of Metformin Hydrochloride and Canagliflozin at 50%, 100% and 150% was added to a pre quantified sample solution and injected into the HPLC system. The mean percentage recovery of Metformin Hydrochloride and Canagliflozin at each level was calculated and the results were presented in (Table 4 and 5).

Preparation of pre quantified sample solution for accuracy studies

Tablet powder equivalent to 200mg of Metformin Hydrochloride and 20mg of Canagliflozin were taken into 100ml clean dry volumetric flask and diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent and was filtered through 0.45 ìm nylon membrane filter. Further pipette out 1ml from the above Metformin Hydrochloride and Canagliflozin sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin.

Preparation of standard solution of Metformin Hydrochloride and Canagliflozin for accuracy studies

Standard stock solutions of Metformin Hydrochloride and Canagliflozin were prepared by dissolving 200mg of Metformin Hydrochloride and 20mg of Canagliflozin in100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 ìm nylon membrane filter and degassed by sonicator to get the concentration of 2000µg/ml of Metformin Hydrochloride and 200µg/ml of Canagliflozin.

Preparation of 50% standard solution

From the standard stock solution of 2000µg/ml of Metformin Hydrochloride and 200µg/ml of Canagliflozin further pipette 0.5ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 100µg/ml of Metformin Hydrochloride and 10µg/ml of Canagliflozin.

Preparation of 100% standard solution

From the standard stock solution of 2000µg/ml of Metformin Hydrochloride and 200µg/ml of Canagliflozin further pipette 1ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin.

Preparation of 150% standard solution

From the standard stock solution of 2000µg/ml of Metformin Hydrochloride and 200µg/ml of Canagliflozin further pipette 1.5ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 300µg/ml of Metformin Hydrochloride and 30µg/ml of Canagliflozin.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%.

Precision studies for Metformin Hydrochloride and Canagliflozin

Method precision (Repeatability)

Tablet powder equivalent to 200mg of Metformin Hydrochloride and 20mg of Canagliflozin were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent and was filtered through 0.45 ìm nylon membrane filter. Further pipette out 1ml from the above Metformin Hydrochloride and Canagliflozin sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin. A homogenous sample of a single batch is analysed six times and was checked whether the method is giving consistent results. The %RSD for the assay of six replicate injections was calculated as mentioned in (Table 6).

Acceptance Criteria

The % RSD for the assay of six sample injections should not be more than 2%.

System precision

The system precision was carried out to ensure that the analytical system is working properly. The standard preparation concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin was injected six times into the HPLC system and the %RSD for the area of six replicate injections was calculated as mentioned in (Table 7).

Acceptance Criteria

The % RSD for the peak area of six standard injections should not be more than 2%.

Intermediate precision/ruggedness

The intermediate precision (also known as Ruggedness) of the method was evaluated by performing precision on different laboratories by different analysts and different days. The sample preparation concentration of 200µg/ml of Metformin Hydrochloride and 20µg/ml of Canagliflozin was injected six times into the HPLC system and the %RSD for the assay of six replicate injections was calculated as mentioned in (Table 8).

Acceptance Criteria

The % RSD for the assay of six sample injections should not be more than 2%.

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

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3×SD/S and 10×SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOD of Metformin Hydrochloride and Canagliflozin was calculated and shown in (Table 9). The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Metformin Hydrochloride and Canagliflozin was calculated and shown in (Table 9).

Robustness

As part of the Robustness, deliberate change in the flow rate, mobile phase proportion of ±10% and column temperature was made to evaluate the impact on the method. The results reveal that the method is robust. The results are summarized in (Table 10, 11 and 12).

Stability of solution

The %RSD of the assay of Metformin Hydrochloride and Canagliflozin from the solution stability and mobile phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable upto 48hours at room temperature was calculated and shown in (Table 13 and 14).

Forced degradation studies

Acid Degradation Studies

To 1ml of stock solution of Metformin Hydrochloride and Canagliflozin, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^{0}C. The resultant solution was diluted to obtain 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 8) and purity plot of acid degradation for Metformin Hydrochloride and Canagliflozin was shown in (Fig. 14-15).

Alkali Degradation Studies

To 1ml of stock solution of Metformin Hydrochloride and Canagliflozin, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^{0}C.The resultant solution was diluted to obtain 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 9) and purity plot of alkali degradation for Metformin Hydrochloride and Canagliflozin was shown in (Fig. 16-17).

Table 1: Assay of marketed formulation of Metformin Hydrochloride and Canagliflozin

Drug	Invokamet Tablet Label Claim (mg)	Amount Found (mg) (n=6)	% Label Claim ± % RSD (n=6)
Metformin Hydrochloride	500	498.54	99.71±0.31
Canagliflozin	50	49.77	99.55±0.43

Table 2: System suitability parameters for Metformin Hydrochloride and Canagliflozin

Parameter (n=6)	Metformin Hydrochloride	Canagliflozin
Retention Time (Mins)	2.440	3.713
Theoretical plates	4216	12854
Tailing factor	1.36	1.16
Resolution		8.95

Table 3: Linearity data for Metformin Hydrochloride and Canagliflozin

Linearity of Metformin Hydrochloride		Linearity of Canagliflozin	
Concentration(µg/ml)	Peak Area	Concentration(µg/ml)	Peak Area
50	222108	5	183389
100	444674	10	365905
150	673854	15	552301
200	904485	20	731880
250	1136832	25	931837
300	1325355	30	1092733

Oxidative degradation Studies

To 1ml of stock solution of Metformin Hydrochloride and Canagliflozin, 1 ml of 3% Hydrogen peroxide (H_2O_2) was added and the solution was kept for 30 mins at 60°C. For HPLC study, the resultant solution was diluted to obtain 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 10) and purity plot

Table 4: Recovery study data of Metformin Hydrochloride

Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	100	100.04	100.04	Mean=99.84% (n=3)
S_2:50%	100	100.40	100.40	S.D=0.682
S_3:50%	100	99.08	99.08	%RSD=0.68
S_4:100%	200	199.50	99.75	Mean=100.65%(n=3)
S_5:100%	200	202.42	101.21	S.D=0.789
S_6:100%	200	202	101.00	%RSD=0.78
S_7:150%	300	298.01	99.34	Mean=99.45%(n=3)
S_8:150%	300	299.33	99.78	S.D=0.291
S_9 :150%	300	297.70	99.23	%RSD=0.29

Table 5: Recovery study data of Canagliflozin

Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	10	10.01	100.11	Mean=100.74%(n=3)
S_2:50%	10	10.03	100.39	S.D=0.864
S_3:50%	10	10.17	101.73	%RSD=0.85
S_4:100%	20	20.04	100.20	Mean=100.06%(n=3)
S_5:100%	20	20.06	100.35	S.D=0.367
S_6:100%	20	19.93	99.65	%RSD=0.36
S_7:150%	30	30.09	100.33	Mean=99.95%(n=3)
S_8:150%	30	30.04	100.16	S.D=0.502
S_9 :150%	30	29.81	99.39	%RSD=0.5

Table 6: Method precision data for Metformin Hydrochloride and Canagliflozin

S.	Metformin Hydrochloride				Canagliflozin			
No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay
1	200	2.438	896027	99.35	20	3.713	734744	99.73
2	200	2.438	898822	99.66	20	3.714	739030	100.31
3	200	2.439	897108	99.47	20	3.717	730041	99.09
4	200	2.439	901800	100.00	20	3.717	732051	99.37
5	200	2.44	903334	100.17	20	3.718	733069	99.50
6	200	2.443	898203	99.60	20	3.732	731465	99.29
Average	2.4395	899215.7	99.71	Average	3.7185	733400	99.55	
SD	0.001871	2808.194	0.311	SD	0.006892	3176.8	0.43	
%RSD	0.076	0.31	0.31	%RSD	0.18	0.43	0.43	

Table 7: System precision data for Metformin Hydrochloride and Canagliflozin

S. No.	Metformin Hydrochloride			Canagliflozin		
	Conc. (ig/ml)	Retention time(min)	Peak Area	Conc. (ig/ml)	Retention time (min)	Peak Area
1	200	2.438	898066	20	3.709	735334
2	200	2.438	897132	20	3.710	733928
3	200	2.438	895528	20	3.713	735524
4	200	2.44	900003	20	3.715	730296
5	200	2.44	896525	20	3.718	734138
6	200	2.444	902170	20	3.727	733405
Average	2.439667	898237	Average	3.715	733771	
SD	0.002338	2453.9	SD	0.006593	1891.29	
%RSD	0.09	0.27	%RSD	0.18	0.26	

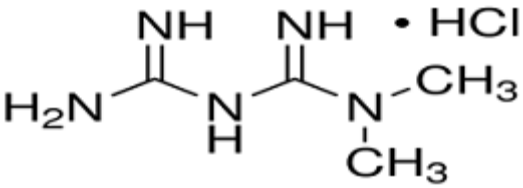

Fig. 1: Chemical structure of Metformin Hydrochloride

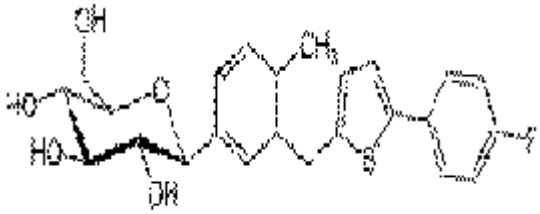

Fig. 2: Chemical structure of Canagliflozin

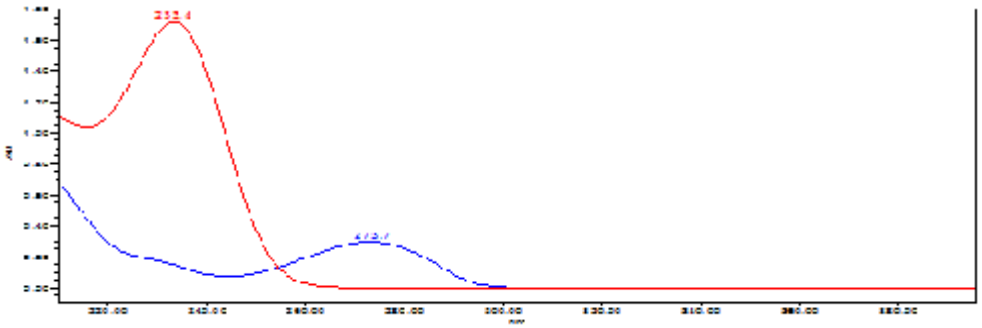

Fig. 3: Isosbestic point of Metformin Hydrochloride and Canagliflozin at 254nm

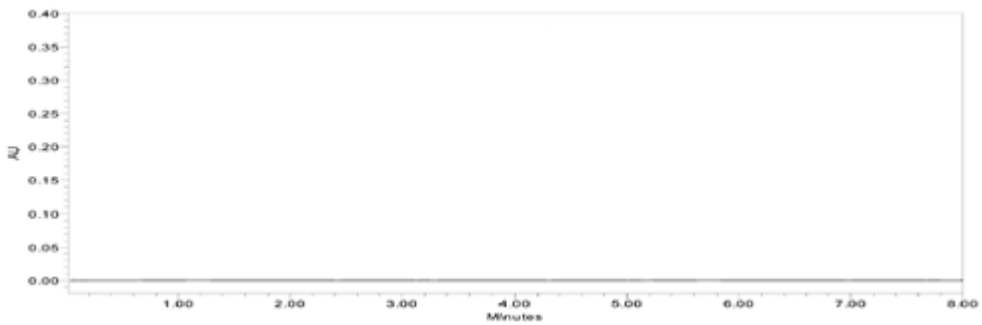

Fig. 4: Chromatogram of blank

of oxidative degradation for Metformin Hydrochloride and Canagliflozin was shown in (Figure 18 and 19).

Thermal Degradation Studies

The standard drug solution was placed in oven at 105^{0}C for 6hrs to study dry heat degradation. For HPLC study, the resultant solution was diluted

Table 8: Ruggedness data for Metformin Hydrochloride and Canagliflozin

	Ruggedness Data for Metformin Hydrochloride							
	Laboratory-1 (% Assay)-HPLC-1				**Laboratory-2 (% Assay)-HPLC-2**			
	Analyst-1		**Analyst-2**		**Analyst-1**		**Analyst-2**	
Conc. (µg/ml)	**Day-1**	**Day-2**	**Day-1**	**Day-2**	**Day-1**	**Day-2**	**Day-1**	**Day-2**
200	99.30	99.42	100.04	99.32	99.17	99.72	100.08	100.16
200	99.64	99.50	99.62	99.51	99.60	99.78	99.97	100.09
200	99.40	99.41	99.56	99.44	99.56	99.65	100.27	99.20
200	100.01	99.66	99.67	99.19	99.70	99.77	99.98	100.01
200	100.19	99.72	99.73	99.62	99.72	100.12	99.95	99.59
200	99.09	99.66	99.29	99.22	99.59	99.71	99.97	99.95
Average	99.61	99.56	99.65	99.38	99.56	99.79	100.04	99.83
SD	0.426	0.135	0.244	0.169	0.200	0.168	0.123	0.368
%RSD	0.43	0.14	0.24	0.17	0.20	0.17	0.12	0.37
	Intermediate precision within-laboratories variations (n=24)							
	Laboratory-1 (% Assay)-HPLC-1				**Laboratory-2 (% Assay)-HPLC-2**			
	Average		**99.55**		**Average**		**99.805**	
	SD		0.243		SD		0.214	
	%RSD		0.24		%RSD		0.21	
	Reproducibility between laboratories (n=48) (% Assay)							
	Average		99.68					
	SD		0.228					
	%RSD		0.22					
	Ruggedness Data for Canagliflozin							
20	99.71	99.86	99.76	99.69	99.26	99.26	99.30	99.28
20	100.07	99.68	99.56	99.38	99.41	99.32	99.79	99.26
20	99.83	99.56	100.02	99.51	99.56	99.24	99.34	99.39
20	99.42	100.01	100.21	99.68	100.10	99.30	100.05	99.48
20	99.75	100.15	99.53	100.14	99.56	100.20	99.68	99.75
20	99.71	99.68	99.38	100.21	99.27	99.26	99.25	100.30
Average	99.75	99.82	99.74	99.77	99.53	99.43	99.57	99.58
SD	0.21	0.23	0.32	0.34	0.31	0.38	0.32	0.40
%RSD	0.21	0.23	0.32	0.34	0.31	0.38	0.32	0.40
	Intermediate precision within-laboratories variations (n=24)							
	Laboratory-1 (% Assay)-HPLC-1				**Laboratory-2 (% Assay)-HPLC-2**			
	Average		99.77		Average		99.52	
	SD		0.275		SD		0.352	
	%RSD		0.27		%RSD		0.35	
	Reproducibility between laboratories (n=48) (% Assay)							
	Average		99.64					
	SD		0.313					
	%RSD		0.31					

Table 9: Summary of validation parameter for Metformin Hydrochloride and Canagliflozin

Parameters	RP-HPLC method			
	Metformin Hydrochloride		Canagliflozin	
Linearity range (µg/ml)	50-300		5-30	
Slope	4475		36722	
Intercept	1187		320.3	
Correlation coefficient	0.999		0.999	
LOD (µg/ml)	0.27		0.01	
LOQ (µg/ml)	0.83		0.04	
Method Precision (% RSD, n=6)	0.31		0.43	
System precision (% RSD, n=6)	0.27		0.26	
Ruggedness (% RSD, n=24)	Lab-1	Lab-2	Lab-1	Lab-2
	0.24	0.21	0.27	0.35
Reproducibility (% RSD, n=48)	0.22	0.31		
% Accuracy	99.45-100.65	99.95-100.74		
Robustness (% RSD, n=6)	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
	0.28	0.05	0.56	0.5
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	1.4	0.41	0.58	0.61
	Less Temperature	More Temperature	Less Temperature	More Temperature
	1.3	0.32	0.74	0.59

Table 10: Summary of robustness (change in flow rate) for Metformin Hydrochloride and Canagliflozin

Drug	Change in Flow rate (ml/min)	Retention Time (Mins)	Change in flow Rate (0.9 ml/min to 1.1 ml/min) Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Metformin	0.9	2.727	999154	2877.2	0.28	4027	1.48
Hydrochloride	1.0	2.439	898237	2453.8	0.27	4216	1.36
	1.1	2.196	801763	422.796	0.05	3526	1.45
Canagliflozin	0.9	4.142	782675	4422.53	0.56	12240	1.19
	1.0	3.715	733771	1891.29	0.26	12854	1.15
	1.1	3.335	638326	3195.37	0.5	11010	1.19

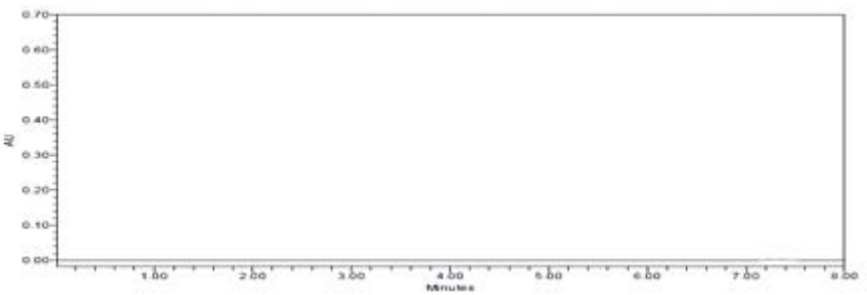

Fig. 5: Chromatogram of placebo

Table 11: Summary of robustness (change in mobile phase) for Metformin Hydrochloride and Canagliflozin

Drug	Change in Mobile Phase	Retention Time (Mins)	Change in mobile phase (0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile) (68:32 v/v to 62:38v/v)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Metformin Hydrochloride	10% less Organic (68:32 v/v)	2.426	912558	13209.89	1.4	3861	1.48
	Actual (65:35 v/v)	2.439	898237	2453.8	0.27	4216	1.36
	10% more Organic (62:38v/v)	2.431	886527	3687	0.41	3887	1.47
Canagliflozin	10% less Organic (68:32 v/v)	3.626	724007	4201.11	0.58	11949	1.19
	Actual (65:35 v/v)	3.715	733771	1891.29	0.26	12854	1.15
	10% more Organic (62:38v/v)	3.689	716425	4385.49	0.61	11538	1.19

Table 12: Summary of robustness (change in column temperature) for Metformin Hydrochloride and Canagliflozin

Drug	Change in column temperature	Retention Time (Mins)	Change in column temperature (28°C to 32°C)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Metformin Hydrochloride	28°C	2.426	911899	11887.64	1.3	3861	1.48
	Actual temperature (30°C)	2.439	898237	2453.8	0.27	4216	1.36
	32°C	2.196	802965	2575.56	0.32	3525	1.44
Canagliflozin	28°C	3.626	720694	5344.83	0.74	11963	1.18
	Actual temperature (30°C)	3.715	733771	1891.29	0.26	12854	1.15
	32°C	3.335	636649	3808.48	0.59	11017	1.19

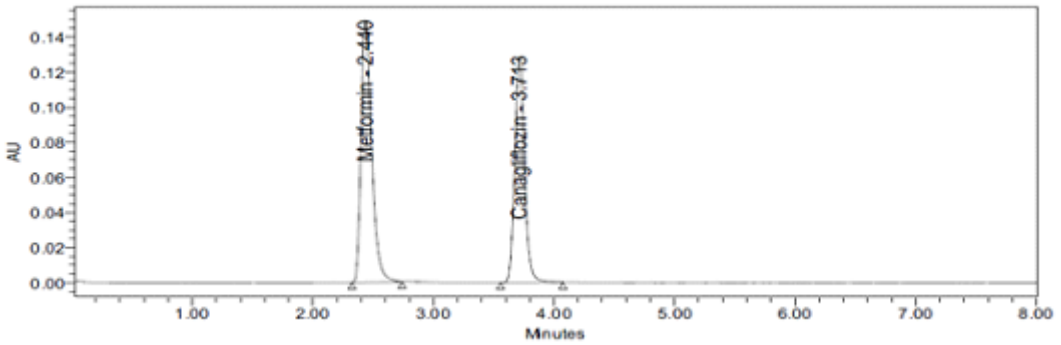

Fig. 6: Standard chromatogram of Metformin Hydrochloride and Canagliflozin

Table 13: Summary of solution stability-effect of pH of mobile phase (0.01M Ammonium acetate and Acetonitrile (65:35, v/v)) (pH adjusted to 3.5 with orthophosphoric acid) for Metformin Hydrochloride for 48 hours at room temperature

S. No.	Solution stability for Metformin Hydrochloride					
	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	200	2.438	898066	99.58	4214	1.35
2	200	2.438	897132	99.48	4124	1.36
3	200	2.438	895511	99.30	4146	1.37
4	200	2.440	900003	99.80	4146	1.37
5	200	2.440	896525	99.41	4183	1.36
6	200	2.444	902170	100.04	4185	1.37
	Average	2.439	898234.5	99.60	4166	1.36
	SD	0.00233809	2457.62054	0.274	33.2184688	0.00816497
	%RSD	0.09	0.27	0.27	0.7	0.59

Table 14: Summary of solution stability-effect of pH of mobile phase (0.01M Ammonium acetate and Acetonitrile (65:35, v/v)) (pH adjusted to 3.5 with orthophosphoric acid) for Canagliflozin for 48 hours at room temperature

S. No.	Solution stability for Canagliflozin					
	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	20	3.709	728817	99.61	12204	1.14
2	20	3.710	729244	99.66	12260	1.16
3	20	3.713	730140	99.79	12254	1.15
4	20	3.715	727898	99.48	12219	1.15
5	20	3.718	728235	99.53	12255	1.16
6	20	3.727	727799	99.47	12266	1.14
	Average	3.715	728688	99.59	12243	1.15
	SD	0.00659293	900.411776	0.12	25.2190404	0.00894427
	%RSD	0.17	0.12	0.12	0.20	0.77

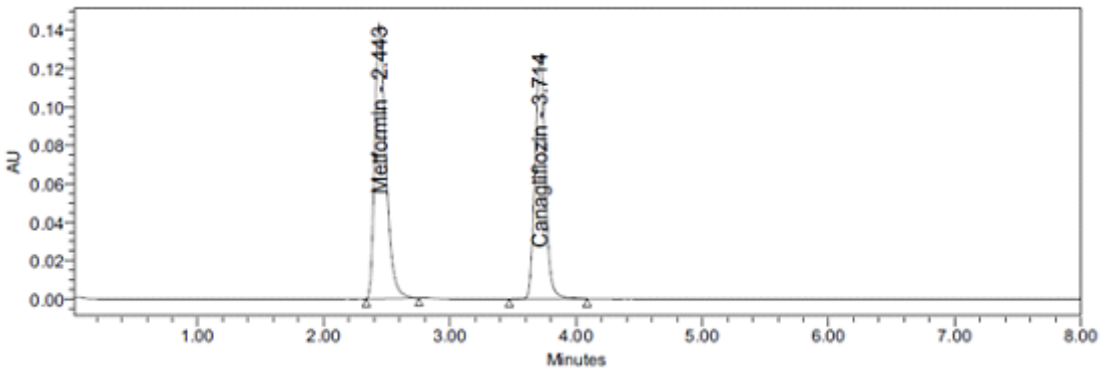

Fig. 7: Sample chromatogram of Metformin Hydrochloride and Canagliflozin

to 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 11) and purity plot of thermal degradation for Metformin Hydrochloride and Canagliflozin was shown in (Figure 20 and 21).

Photolytic degradation studies

The photochemical stability of the drug was also studied by exposing the drug solution to UV light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 12) and purity plot of photolytic degradation for Metformin Hydrochloride and Canagliflozin was shown in (Figure 22 and 23).

Water Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 200µg/ml and 20µg/ml solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was

Table 15: Forced degradation data of Metformin Hydrochloride and Canagliflozin in different degradation conditions

Degradation condition	Forced degradation data of Metformin Hydrochloride						
	Retention time	Area	% Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	2.433	849907	55.68	0.275	0.978	5459	1.5
Alkaline hydrolysis	2.431	853423	54.83	0.224	0.540	3846	1.5
Oxidative degradation	2.594	830313	22.85	0.192	0.204	8377	1.3
Thermal degradation	2.431	879803	55.14	0.259	0.545	3832	1.5
Photolytic degradation	2.433	881806	54.84	0.252	0.525	3897	1.5
Water degradation	2.431	885972	55.82	0.209	0.617	3885	1.5
	Forced degradation data of Canagliflozin						
Acid hydrolysis	3.692	696059	44.31	0.156	0.365	12389	1.2
Alkaline hydrolysis	3.688	705872	45.16	0.131	0.284	12113	1.2
Oxidative degradation	3.690	705279	38.89	0.124	0.283	11823	1.2
Thermal degradation	3.688	712117	44.86	0.130	0.281	12128	1.2
Photolytic degradation	3.692	715119	45.16	0.136	0.289	11369	1.2
Water degradation	3.701	721144	44.18	0.120	0.290	11835	1.2

Degradation condition	Drug Recovered (%)		Drug Decomposed (%)	
	Metformin Hydrochloride	Canagliflozin	Metformin Hydrochloride	Canagliflozin
Standard	100	100	100	100
Acid hydrolysis	95.22	95.63	4.78	4.37
Alkaline hydrolysis	95.61	96.98	4.39	3.02
Oxidative degradation	93.02	96.90	6.98	3.10
Thermal degradation	98.57	97.84	1.43	2.16
Photolytic degradation	98.79	98.25	1.21	1.75
Water degradation	99.26	99.08	0.74	0.92

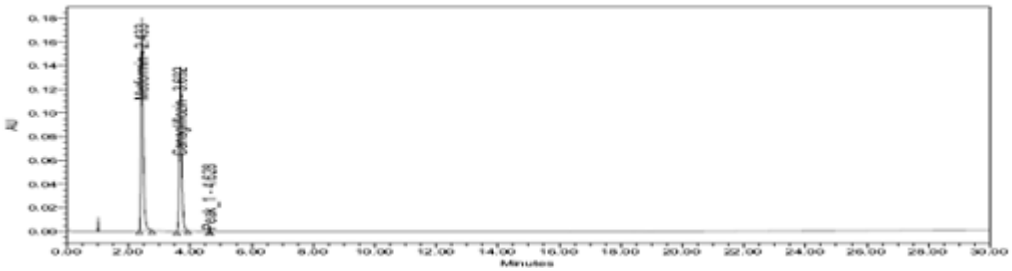

Fig. 8: Chromatogram of acid hydrolysis for Metformin Hydrochloride and Canagliflozin

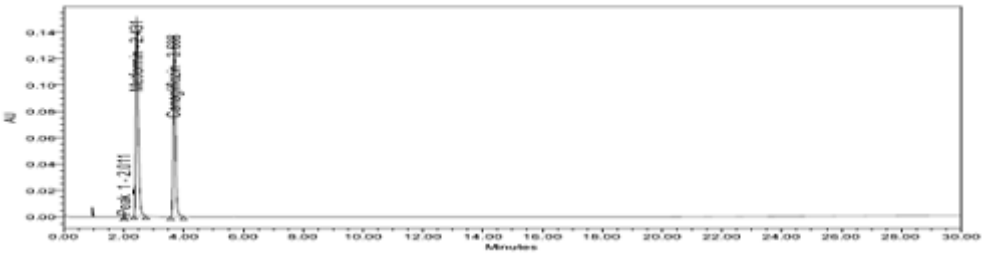

Fig. 9: Chromatogram of alkali hydrolysis for Metformin Hydrochloride and Canagliflozin

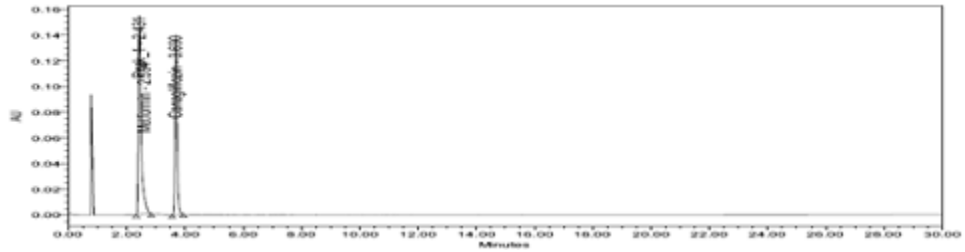

Fig. 10: Chromatogram of oxidative degradation for Metformin Hydrochloride and Canagliflozin

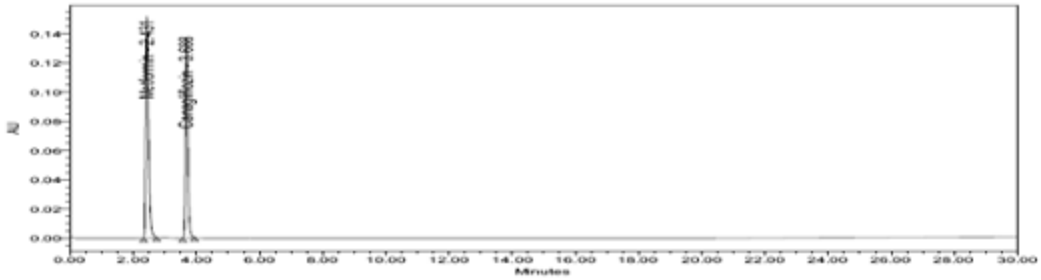

Fig. 11: Chromatogram of thermal degradation for Metformin Hydrochloride and Canagliflozin

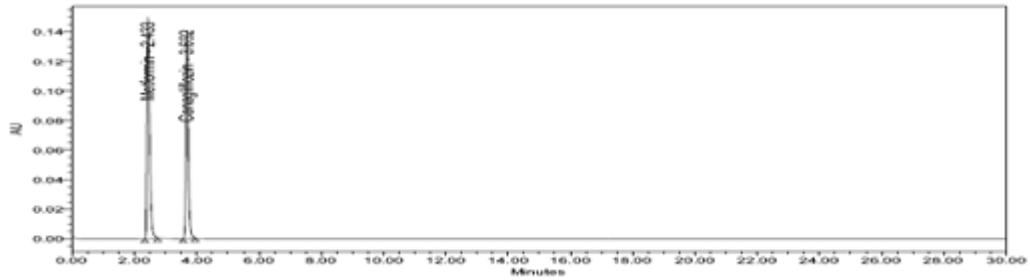

Fig. 12: Chromatogram of photolytic degradation for Metformin Hydrochloride and Canagliflozin

shown in (Figure 13) and purity plot of water degradation for Metformin Hydrochloride and Canagliflozin was shown in (Figure 24 and 25).

RESULTS AND DISCUSSION

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Metformin Hydrochloride and Canagliflozin were obtained with a mobile phase containing a mixture of 0.01M Ammonium acetate (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v) was delivered at a flow rate of 1ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 254nm based on peak area. The retention time of Metformin Hydrochloride and Canagliflozin was found to be 2.440min and 3.713min respectively with resolution of 8.95was shown in (Figure 6). Linearity was established for Metformin Hydrochloride and Canagliflozin in the range of 50-300µg/ml for Metformin Hydrochloride and 5-30µg/ml for Canagliflozin with correlation coefficients (r^2=0.999) and the percentage recoveries were between 99.45 % to 100.65% and 99.95% to 100.74% for Metformin Hydrochloride and Canagliflozin respectively, which indicate accuracy of the proposed method. The % RSD values of accuracy for Metformin Hydrochloride and

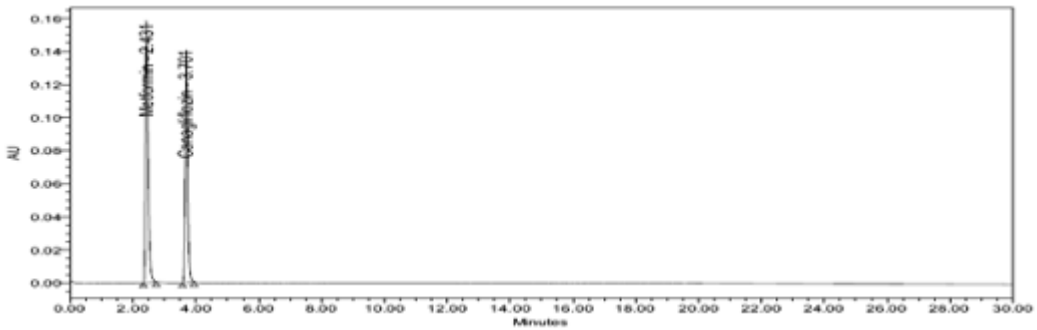

Fig. 13: Chromatogram of water degradation for Metformin Hydrochloride and Canagliflozin

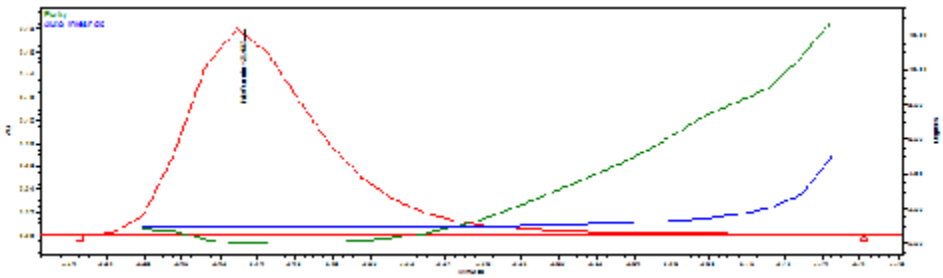

Fig. 14: Purity plot of acid hydrolysis for Metformin Hydrochloride

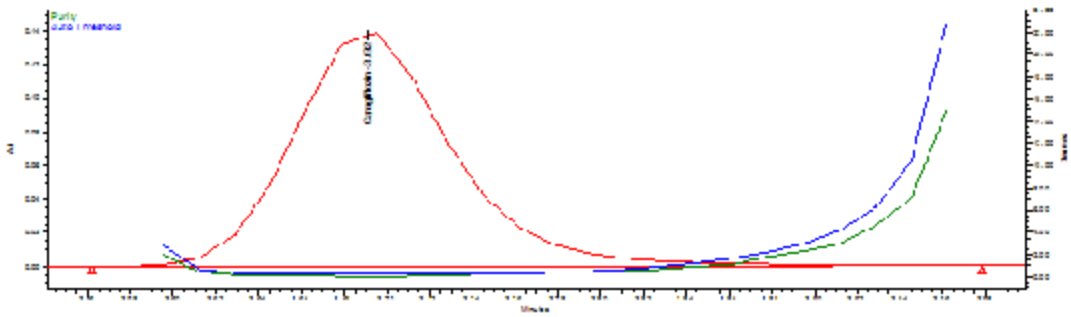

Fig. 15: Purity plot of acid hydrolysis for Canagliflozin

Canagliflozin were found to be < 2 %. The % RSD values of method precision are 0.31% and 0.43% for Metformin Hydrochloride and Canagliflozin respectively and % RSD values of system precision are 0.27% and 0.26% for Metformin Hydrochloride and Canagliflozin. The % RSD values of reproducibility are 0.22% and 0.31% for Metformin Hydrochloride and Canagliflozin respectively, reveal that the proposed method is precise. LOD values for Metformin Hydrochloride and Canagliflozin were

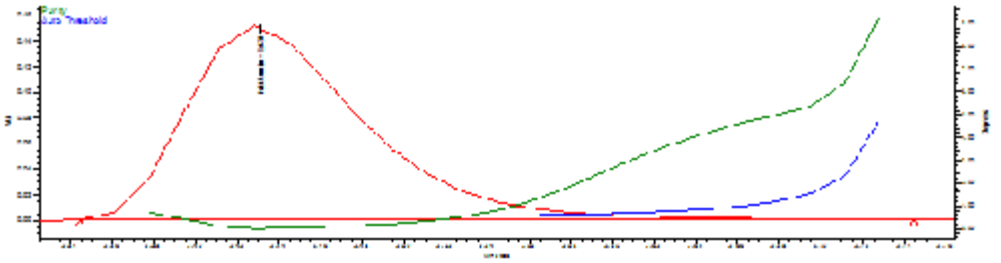

Fig. 16: Purity plot of alkali hydrolysis for Metformin Hydrochloride

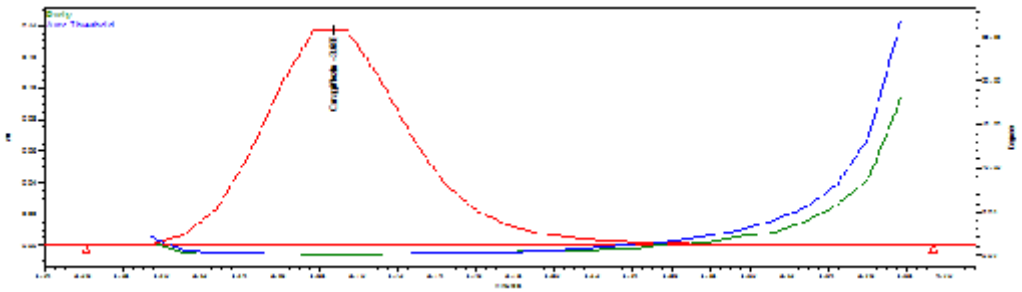

Fig. 17: Purity plot of alkali hydrolysis for Canagliflozin

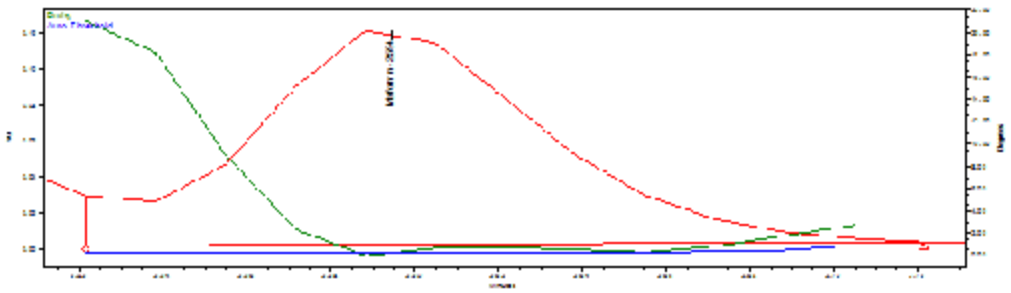

Fig. 18: Purity plot of oxidative degradation for Metformin Hydrochloride

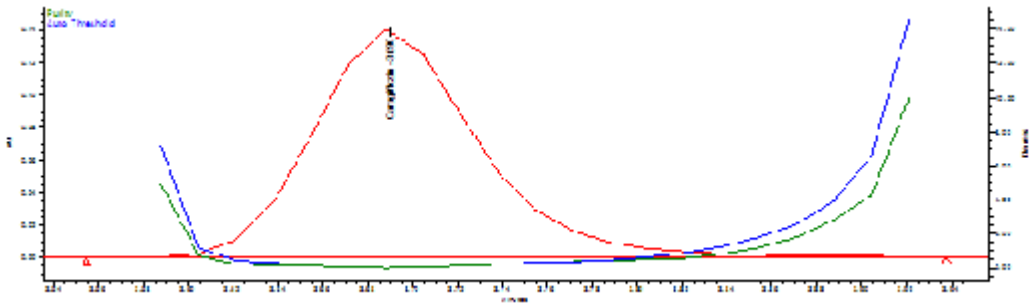

Fig. 19: Purity plot of oxidative degradation for Canagliflozin

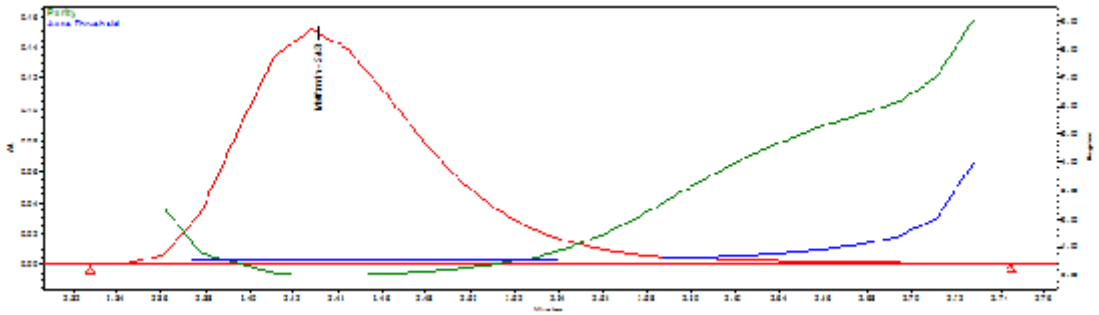

Fig. 20: Purity plot of thermal degradation for Metformin Hydrochloride

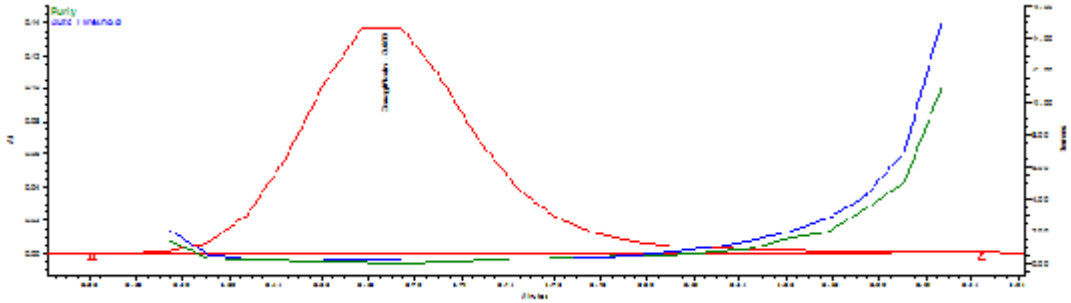

Fig. 21: Purity plot of thermal degradation for Canagliflozin

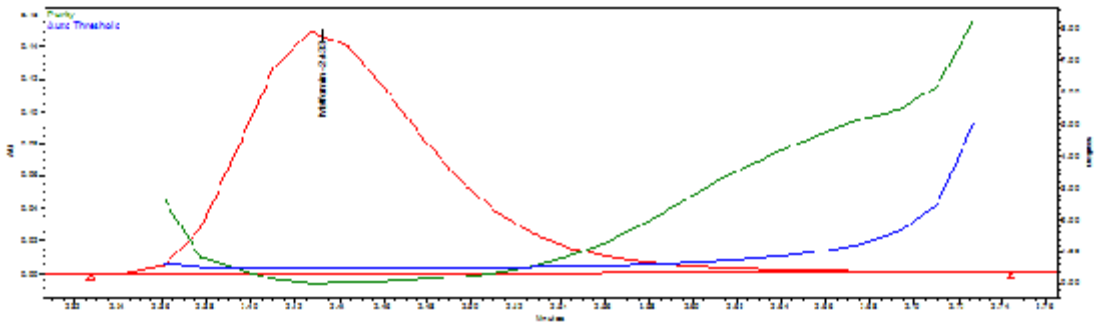

Fig. 22: Purity plot of photolytic degradation for Metformin Hydrochloride

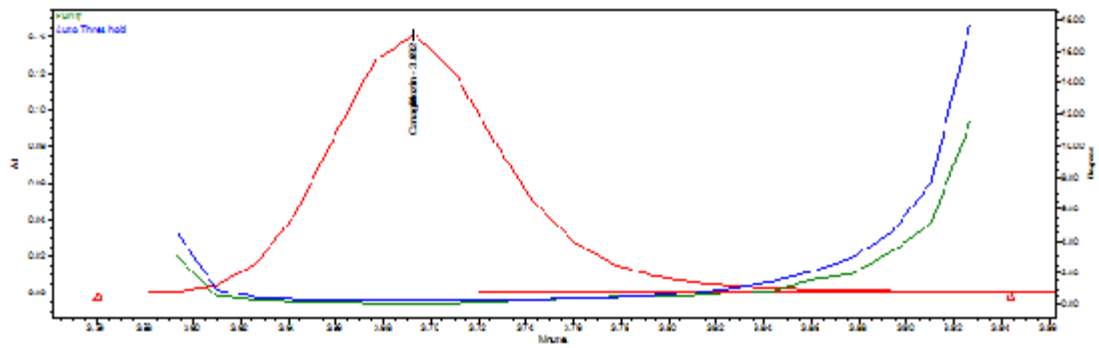

Fig. 23: Purity plot of photolytic degradation for Canagliflozin

found to be 0.27µg/ml and 0.01µg/ml respectively and LOQ values for Metformin Hydrochloride and Canagliflozin were found to be 0.83µg/ml and 0.04µg/ml respectively was shown in (Table 9). The % RSD values of robustness studies were found to be < 2% reveal that the method is robust enough was shown in (Table 10, 11 and 12). These data show that the proposed method is specific and sensitive for the determination of Metformin Hydrochloride and Canagliflozin. The results of system suitability testing are given in (Table 2).

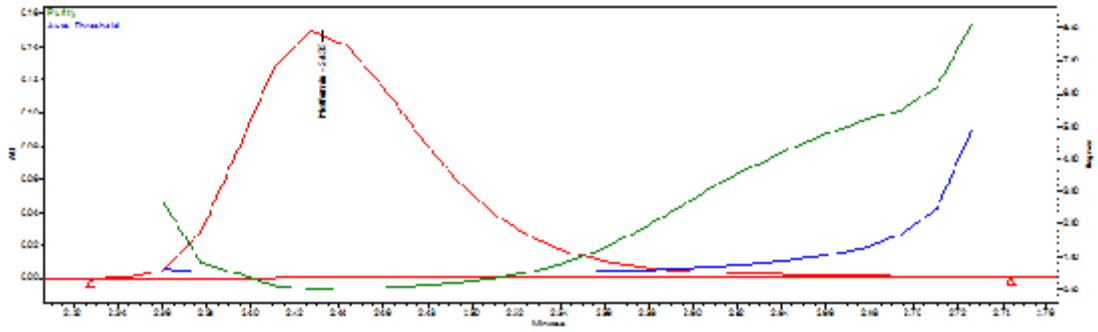

Fig. 24: Purity plot of water degradation for Metformin Hydrochloride

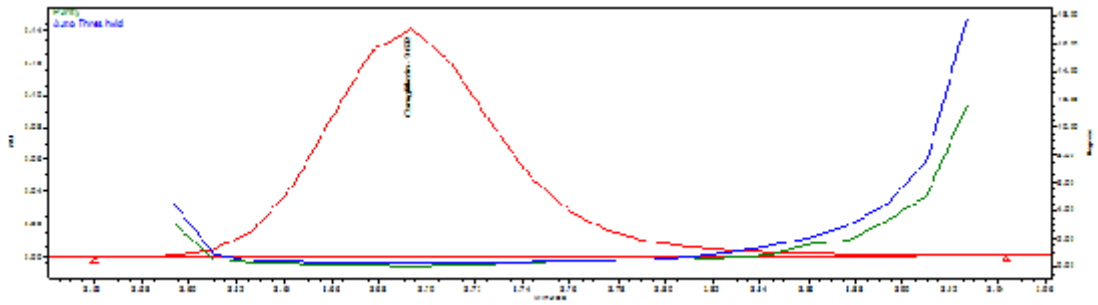

Fig. 25: Purity plot of water degradation for Canagliflozin

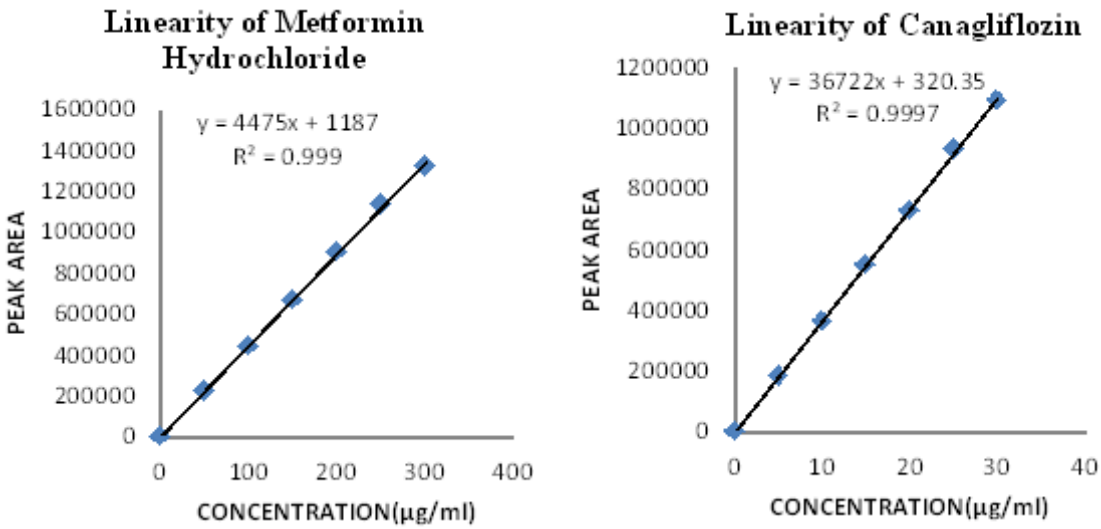

Fig. 26: Linearity graph of Metformin Hydrochloride and Canagliflozin

CONCLUSION

RP-HPLC method for the simultaneous estimation of Metformin Hydrochloride and Canagliflozin in their combine dosage form was established and validated as per the ICH guidelines. Linearity was achieved for Metformin Hydrochloride and Canagliflozin in the range of 50-300µg/ml for Metformin Hydrochloride and 5-30µg/ml for Canagliflozin with correlation coefficients (r^2=0.999). The percentage recoveries of Metformin Hydrochloride and Canagliflozin were achieved in the range of 98-102% which was within the acceptance criteria. The percentage RSD was NMT 2 % which proved the precision of the developed method. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation and water. Metformin Hydrochloride and Canagliflozin are more sensitive towards oxidative degradation condition and moderate degradation towards acidic, alkaline and very much resistant towards thermal, photolytic and water degradations which was shown in (Table 15). No interference from any components of pharmaceutical dosage form or degradation products was observed and the method has been successfully used to perform long term and accelerated stability studies of Metformin Hydrochloride and Canagliflozin formulations. Hence it can be used for the hyphenated instrumental analysis of Metformin Hydrochloride and Canagliflozin in their bulk and combine dosage form.

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REFERENCES

1. Bailey, C.J.; Turner, R.C. *N Engl J Med.,* **1996**, *334*, 574-579.
2. Kim, Y.D.; Park, K.G.; Lee, Y.S.; Park, Y.Y.; Kim, D.K.; Nedumaran, B.; Jang, W.G.; Cho, W.J.; Ha, J.; Lee, I.K.; Lee, C.H.; Choi, H.S. *Diabetes*, **2008**, *57*, 306-314.
3. Viollet, B.; Guigas, B.; Sanz Garcia, N.; Leclerc, J.; Foretz, M.; Andreelli, F. *Clin Sci.,* **2012**, *122*, 253-270.
4. Edward Chao, C. *Drugs of the Future*, **2011**, *36*, 351–357.
5. Chellu, S. N.; Malleswara, R.; Mulukutla Suryanarayana, V.; Mukkanti, K. *Sci Pharm.*, **2012**, *80*, 139–152.
6. Valentina, P.; Simone, G.S.; Eunice, K.K.; Eunice Emiko, K.; Yara, P. A.; Kazuo, F.; Cristina Helena dos, R. S. *Journal of Pharmaceutical and Biomedical Analysis,* **2008**, *46*, 143–147.
7. Kar, M.; Choudhury, P.K. *Indian J Pharm Sci.,* **2009**, *71*,318-320.
8. Bhamare, P.C.; Bari, S.B.; Natarajan, S.; Patil, A.A.; Patil, S.H.; Shirode, P.T. *Asian Journal of Biochemical and Pharmaceutical Research,* **2011**, *1*, 115-128.
9. Rashmi Ranjan, S.; Satya Narayana, P.; Susanta Kumar, P.; Kanhu Charana, S. *International Journal of Pharmaceutical & Biological Archives,* **2011**, *2*, 1137-1145.
10. Ramzia El-Bagary, I.; Ehab Elkady, F.; Bassam Ayoub, M. *International journal of Biomedical science*, 2011, *7*, 62-69.
11. Sheela, N.R.; Muthu, S.; Sampath, S.K. *Asian Journal of Chemistry*, **2010**, *22,* 5049-5056.
12. Habib, I.H.I.; Kamel, M. S. *Talanta,* **2003**, *60,* 185-190.
13. Gadape, H.H.; Parikh, K.S. *E-Journal of Chemistry,* **2011**, *8*, 767-781.
14. Muzaffar, I.; Essam, E.; Khalid Al-Rashood, A.; Yousif Asiri, A.; Naser Rezk, L. *Talanta,* **2015**, *132*, 29-36.
15. Edward, P.C.L.; Feng, S.Y. *Journal of Chromatography B*, **2006**, *843,* 94–99.
16. Devineni, D.; Curtin, C.R.; Ariyawansa, J.; Weiner, S.; Stieltjes, H.; Vaccaro, N.; Shalayda, K.; Murphy, J.; Di Prospero, N.A.; Wajs, E. *J Bioequiv.*, **2014**, *6*, 164-173.
17. Shabir, G. A. *J Chromatogr A.* **2003**, *987*, 57–66.