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A Review Study of "Analytical Validation of Stability Indicating Reverse Phase - HPLC for Metformin Hydrochloride-Canagliflozin"

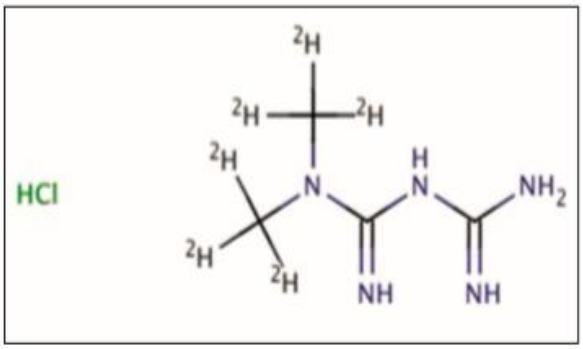
Devendra Singh Chahar, Dr. U S Sharma, Vimal Kumar Sharma

***Abstract*— A new method indicating RP-HPLC has been developed and validated for both Drug Metformin Hydrochloride and Canagliflozin in bulk and dosage forms. The method involves separation Agilent C18 (250x4.6mm) 5µ particle size. The optimized mobile phase consists of Potassium Dihydrogen Phosphate Buffer (pH 3.2 ± 0.1) and Acetonitrile (40:60 v/v) with a flow rate of 1ml/min and UV detection at 240 nm. Retention time was 2.209 min (Metformin Hydrochloride), 4.799 min (Canagliflozin). Linearity range was 100-500 ug/ml (Metformin Hydrochloride), 10-50ug/ml (Canagliflozin). Accuracy Precision was in the range for Metformin Hydrochloride and Canagliflozin. LOD and LOQ are 0.15ug/ml and 0.46 ug/ml for Metformin Hydrochloride, 0.19ug/ml and 0.58ug/ml for Canagliflozin. The method developed is more sensitive, accurate and precise than the methods reported earlier. Retention time and run time were also less and hence the method is economical.**

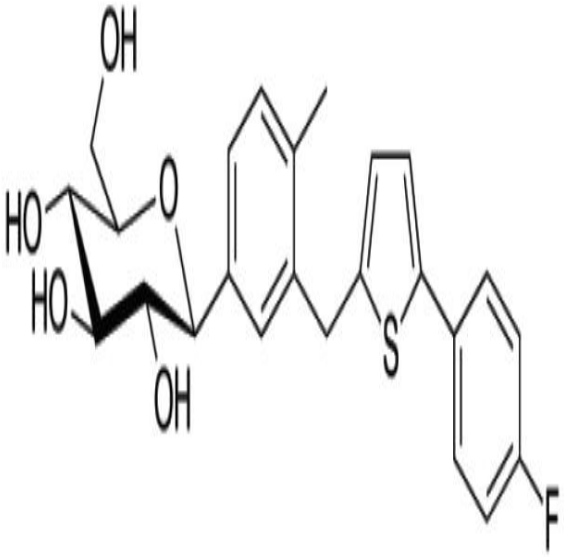
***Index Terms*— Metformin Hydrochloride , Canagliflozin , RP-HPLC Method , ICH Guidelines etc.**

I. INTRODUCTION

Metformin (MET) has a chemical term which is N, N dimethyl imido dicarbonimidic diamide (4 1-Carbamimidamido-N, N-Dimethyl Methanimidamide) is a first line oral pharmacotherapy for the type 2 diabetes. It decreases the production of hepatic glucose to a large extent; hence, improves glycemic control. It also increases insulin-mediated uptake of glucose. MET can initiate weight loss so this drug is a choice of obese NIDDM patients.



Canagliflozin(C24H25FO5S) (CANA) has a chemical term which is (2S, 3R, 4R, 5S, 6R)-2-[3-[[5-(4-fluorophenyl) thiophen-2-yl] methyl]-4-methylphenyl]-6-(hydroxymethyl) oxane- 3, 4, 5-triol is a white to off white solid with melting range of 95-105ºC Canagliflozin is first Sodium-glucose co-transporter 2 (SGLT-2) inhibitor is preferred in type 2 diabetes.



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II. EXPERIMENTAL

INSTRUMENTS EMPLOYED

Sr.No.	Instruments	Make & Model
1.	Digital balance	Wenser
2.	pH Meter	Digital pH meter Instrument India
3.	Sonicator	Ultra wave, instrument India
4.	Membrane filter	Nylon membrane filter (0.45µ)
5.	HPLC SHIMADZU-LC 20 AT	
	Software used	LC Solution
	Detector	UV-detector
	Analytical column	Agilent C_{18} (250x4.6mm) 5µ
6.	UV-SHIMADZU (UV -Visible Spectrophotometer)	
	Instrument Model	UV-1800
	Instrument Type	UV -1800PC Series
	Software Name & Version	UV prove 2.21 Version

CHEMICALS REQUIRED

Metformin working reference standard	AR grade
Canagliflozin working reference Standard	AR grade
Potassium dihydrogen ortho phosphate	AR grade
Acetonitrile	HPLC grade
Ortho phosphoric acid	HPLC grade
Water milli-Q grade	HPLC grade
Methanol	HPLC grade
tablets brand used: Invokamet (Purchase From Market)	(500 mg of Metformin and 50mg of Canagliflozin) Manufacturer-Janssen Pharma , Canada

Optimization of mobile phase:

Separation of both the drugs was tried using the following combination of mobile phases.

Table No. 1 Method development trails

Sr. No.	Mobile phase	Ratio (v/v)	Elution of peak
1.	Buffer : Acetonitrile	50 : 50	Not proper separation
2.	Buffer : Acetonitrile	80: 20	Not proper separation
3.	Buffer : [Sodium Acetate: Methanol : Acetonitrile 5:40 : 55 v/v]	55:45	Separation of peaks
4.	Buffer : [Orthophosphoric acid Acetonitrile : 20 : 80 v/v]	20 : 80	Separation of peaks
5.	Buffer : [Acetonitrile : Orthophosphoric acid 50:50 v/v]	50 : 50	Separation of peaks
6.	**Buffer : [Acetonitrile : Phosphate Buffer 60:40 v/v] pH adjust with** Orthophosphoric acid	**60 : 40 (pH-3.2)**	**Good Separation**

Out of 6 trials the 6th trial was selected for further studies because when compared to other trails

5th trial was found less in retention time due to the ratio or organic solvent in mobile phase.

Optimized Conditions:

The following optimized parameters were used as a final method for the simultaneous estimation of Metformin and Canagliflozin.

Instrument : SHIMADZU – LC 2010
Column : : Agilent C18 (250x4.6mm) 5µ
Column Oven Temperature : 30º C
Wave length : 240 nm
Flow rate : 1.0ml/min
Injection Volume : 20µl
Runtime : 8 minutes

Mode of Operation : Reverse phase
Mobile Phase (Solvent ratio) : 60:40 v/v of Solvent A: B
Solvent A : (Potassium dihydrogen phosphate Buffer - 60 v/v)
Solvent B : (Acetonitrile– 40 v/v)

Preparation of Standard Stock solution:

Metformin: Weigh and transfer accurately 100 mg of Metformin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of Diluent and sonicate to dissolve.

Canagliflozin: Weigh and transfer accurately 100 mg of Canagliflozin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of Diluent and sonicate to dissolve. Further diluted as per different dilution.

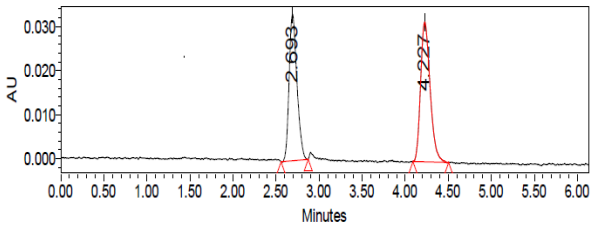
Sample Solution : Weighed and finely powdered not less than 20 tablets. Transferred an accurately weighed portion of the powder of Metformin about 500mg and Canagliflozin about 50 mg in 100ml volumetric flak, added 100ml of Diluent phase sonicated for 30 minutes. Make up the volume with Diluent. Mixed well and filtered through 0.45µ nylon filter paper discarded first few ml of the filtrate. Further diluted as per different dilution

Procedure: Injected separately 20µl of the standard preparation into the equilibrated HPLC system in replicate and measure the response of the major peak due to Metformin and Canagliflozin. Then injected 20µl of the sample preparation in duplicate and measured the response of the major peak due to Metformin and Canagliflozin. Calculate the content of Metformin and Canagliflozin.

III. RESULTS AND DISCUSSION:

Optimization of Chromatographic Conditions:

Initially, the reverse phase liquid chromatography separation was tried to develop using various ratios of Methanol and Water, Acetonitrile and Water as mobile phases, in which both the drugs did not respond properly, and the resolution was also poor. The organic content of the mobile phase was also investigated to optimize the separation of both drugs. Proper selection of the method depends upon the nature of the sample (ionic or ionisable or neutral molecule), its molecular weight, and solubility. Metformin Hydrochloride and Canagliflozin were dissolved in polar solvent, so the developed method of estimation was carried out on reverse phase high performance liquid chromatography. To develop a rugged and suitable HPLC method for the quantitative determination of Metformin and Canagliflozin, the analytical conditions were selected after the consideration of different parameters such as diluents, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition, and other chromatographic conditions. Preliminary trials were taken with different composition of buffer and organic phase of mobile phases with pH range of 2.5–5. The column selection has been done by backpressure, resolution, peak shape, theoretical plates, and day-to-day reproducibility of the retention time and resolution To improve the tailing factor, the pH of the mobile phase becomes an important factor. between Metformin and Canagliflozin peak. After evaluating all these factors, a Kromosil C_{18} column was found to be giving satisfactory results. The selection of Acetonitrile and buffer were based on chemical structure of both the drugs. The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates, and peak shape of both components. Best results were obtained with 0.1% O-phosphoric acid pH adjusted to 2.8 with sodium hydroxide solution that improved the peak shapes of Metformin Hydrochloride and Canagliflozin. For the selection of organic constituent of mobile phase, Acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Therefore, final mobile phase composition consisting of Acetonitrile: a mixture of buffer - pH 3.2 (0.1% ortho phosphoric acid) (60:40 v/v). Flow rates between 0.5 to 2.0 ml/min were tried. Flow rate of 1ml/min was observed to be enough to get both the drugs eluted within less than 10 min. The column temperature was set at 30 °C. Optimized method was providing good resolution and peak shape for Metformin Hydrochloride and Canagliflozin. Under above described experimental conditions, all the peaks were well defined and free from tailing. The concern of small deliberate changes in the mobile phase composition, flow rates, and column temperature on results were evaluated as a part of testing for methods robustness. At pH: 3.2 both drugs eluted with better separation. Thereafter, buffer: Acetonitrile were taken in the isocratic ratio: %buffer / % Acetonitrile : 10/40, 15/35, 20/40, 35/25, and 40/60 and with a flow rate of 0.5mL/min to 1.0mL/min was employed. ODS 250mm x 4.6 mm, 5 particle size was selected as the stationary phase to improve resolution and the tailing of both peaks was reduced considerably and brought close to 1. To analyze both drugs detection were tried at various wavelengths from 205 nm to 280 nm. Both MET and CANA showed maximum absorption at 240 nm of wavelength and selected as the detection wavelength for PDA detector. The retention times were found to about 2.693 min and 4.227 min for MET and CANA, respectively. The chromatogram obtained was shown in the Fig.



Atypical Chromatogram of Metformin and Canagliflozin

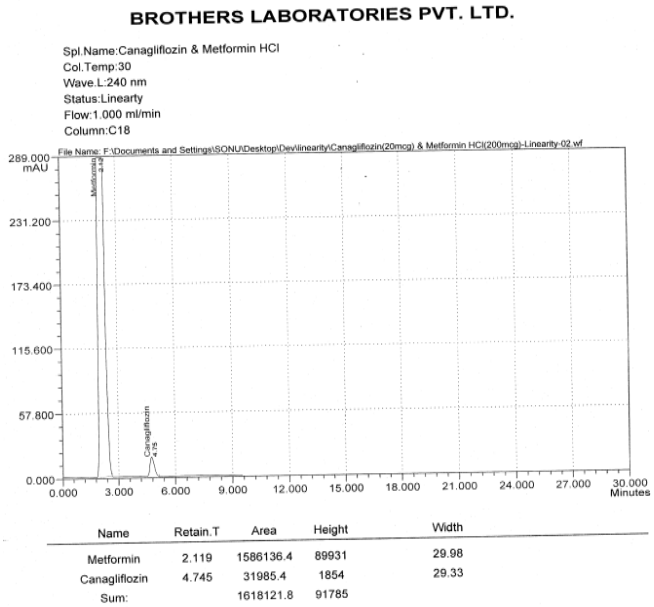
Validation of Method Developed:

The proposed method was validated according to the ICH guidelines for system suitability, specificity, precision, linearity, and robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

System Suitability: The Retention time of Metformin Hydrochloride and Canagliflozin using optimum conditions was 2.68 min and 4.22 min respectively and the theoretical

plate's numbers were >4000 and % RSD of areas of standard injections of Metformin Hydrochloride and Canagliflozin was less than 2. These values are within the acceptable range of United States pharmacopoeia definition and the chromatographic conditions.

Linearity and Range: Linearity was assessed for the two oral anti diabetic drugs at concentration ranges 100-500µg/ml for Metformin Hydrochloride and 10-50µg/ml for Canagliflozin. The Chromatograms of level 2 and level 5 are shown in **Fig. 2** and **Fig. 3**. A linear relationship was established at these ranges between Area under the peak (AUP) and concentration. The results were tabulated in **Table 2.**



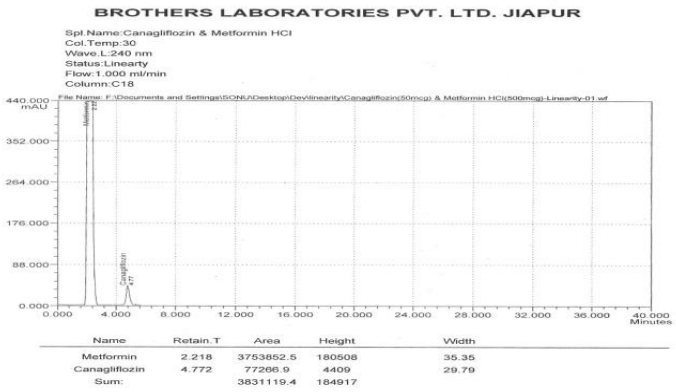


TABLE 2: LINEARITY DATA OF METFORMIN HYDROCHLORIDE AND CANAGLIFLOZIN				
Level	**Concentration of Metformin Hydrochloride (µg/ml)**	**Peak area**	**Concentration of Canagliflozin (µg/ml)**	**Peak area**
1	100	7401833.0	10	17330.8
2	200	1586964.3	20	31920.8
3	300	2223356.5	30	45628.5
4	400	3043045.3	40	61927.2
5	500	3753852.5	50	77136.6

Limit of Detection (LOD) / Limit of Quantitation (LOQ):

The LOD was determine don the basis of signal to noise ratios and was determined using analytical response of three times the background noise. LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. Both LOQ and LOD were calculated on the peak area using the following equations:

LOQ= 10 x N/ B

LOD= 3 x N/ B

The limit of detection and limit of quantification were evaluated by serial dilutions of Metformin Hydrochloride and Canagliflozin stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD and LOQ for MET were 0.15 and 0.46 µg/mL respectively and for CANA were 0.19 and 0.58 µg/mL, respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive.

Precision:

System Precision: System Precision was carried to ensure analytical system is working properly. One dilution of both the drugs in six replicates was injected into HPLC system and was analyzed and the results were found within the acceptance limits (RSD < 2) as shown in the **Table 3**.

Method Precision (Repeatability): Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of sample preparation of Metformin (500µg/mL) and Canagliflozin (50µg/mL) have been analyzed by injecting them into a HPLC column on the same day and on consecutive days. From the results obtained, % RSD was calculated and was found to be within the limits (< 2). The results of precision are given in **Table 4**.

TABLE 3: SYSTEM PRECISION DATA FOR METFORMIN HYDROCHLORIDE AND CANAGLIFLOZIN						
Metformin Hydrochloride				**Canagliflozin**		
Sr. No.	**Concentration (µg/ml)**	**RT (min)**	**Peak Area**	**Concentration (µg/ml)**	**RT (min)**	**Peak Area**
1	500	2.209	3791753	50	4.799	74346
2	500	2.209	3790455	50	4.799	74313
3	500	2.211	3788050	50	4.79	74418
4	500	2.209	3790342	50	4.798	74473
5	500	2.219	3790420	50	4.799	74418
6	500	2.210	3790884	50	4.799	74444
	Average	3790317		Average	74402	
	SD	1229		SD	61	
	% RSD	0.03		% RSD	0.08	

TABLE 4: METHOD PRECISION DATA FOR METFORMIN HYDROCHLORIDE AND CANAGLIFLOZIN						
Metformin Hydrochloride				**Canagliflozin**		
Sr. No.	**Concentration (µg/ml)**	**Peak Area**	**% Assay**	**Concentration (µg/ml)**	**Peak Area**	**% Assay**
1	500	3789260	99.3	50	73026	99.22
2	500	3790455	99.4	50	74136	100.54
3	500	3791731	100.4	50	74289	100.41
4	500	3788057	99.4	50	74506	99.41
5	500	3790420	100.1	50	74292	99.05
6	500	3791753	99.6	50	74346	100.2
	Average	3790279	99.7	Average	74099	99.8
	SD	1435	0.44	SD	539	0.65
	% RSD	0.04	0.45	% RSD	0.73	0.651

Ruggedness: Intermediate precision was accessed injecting sample preparation of Metformin (100 µg/mL) and Canagliflozin (100µg/mL) in six replicates in to HPLC column on the same day and on consecutive days and in different laboratories by different analysts. Results were found within the acceptance limits (RSD < 2) as shown in the **Tables5** and **6** below

TABLE 5: RUGGEDNESS DATA FOR METFORMIN HYDROCHLORIDE								
Laboratory-1 (% Assay)-HPLC-1					**Laboratory-2 (% Assay)-HPLC-2**			
	Analyst 1		Analyst 2		Analyst 1		Analyst 2	
Concentration (µg/ml)	Day 1	Day 2	Day1	Day 2	Day 1	Day 2	Day 1	Day 2
100	99.98	99.98	99.87	99.45	99.83	99.84	100.02	99.86
100	99.76	100.11	99.91	99.92	99.50	99.53	99.42	100.02
100	99.74	99.52	99.76	99.24	99.58	99.81	99.27	99.52
100	99.89	99.86	100.14	99.56	99.45	99.25	99.48	99.54
100	100.29	99.84	100.07	100.23	100.23	100.08	99.59	100.05
100	100.03	100.14	99.91	100.14	99.89	100.12	99.76	99.92
Average	99.94	99.90	99.94	99.69	99.74	99.77	99.59	99.82
SD	0.203	0.226	0.138	0.397	0.295	0.332	0.27	0.23
% RSD	0.20	0.23	0.14	0.40	0.30	0.33	0.27	0.23

TABLE 6: RUGGEDNESS DATA FOR CANAGLIFLOZIN								
Laboratory-1 (% Assay)-HPLC-1					**Laboratory-2 (% Assay)-HPLC-2**			
	Analyst 1		Analyst 2		Analyst 1		Analyst 2	
Concentration (µg/ml)	Day 1	Day 2	Day1	Day 2	Day 1	Day 2	Day 1	Day 2
100	99.42	99.46	99.94	99.76	99.94	99.99	99.91	99.21
100	100.01	100.1	99.66	99.55	99.59	99.55	99.84	99.48
100	99.78	99.89	100.14	99.52	99.44	99.81	99.51	99.42
100	100.1	99.9	99.68	100.13	99.79	99.72	99.89	99.61
100	99.95	100.1	99.76	100.32	99.84	100.08	100.1	99.49
100	100.14	99.89	99.93	99.74	100.1	99.12	99.37	100.21
Average	99.9	99.89	99.85	99.83	99.78	99.71	99.77	99.57
SD	0.267	0.233	0.185	0.321	0.238	0.346	0.274	0.339
% RSD	0.27	0.23	0.19	0.32	0.24	0.35	0.27	0.37

7. Accuracy: The percentage recovery was calculated by preparing standard drug concentrations of Metformin hydrochloride and Canagliflozin with concentration levels of 100 %, 200% and 500%. To pre-analyzed sample solution, a definite concentration of standard drug (10%, 100% & 150 % level) was added and recovery was studied. The results are given in **Tables 7** and **8**.

Table 7 : RECOVERY DATA OF METFORMIN				
Sample name	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% Recovery**	**Statistical Analysis**
S1:100%	100	100.69	100.69	Mean=100.27%
S2:100%	100	101.65	100.65	S.D=0.681
S3:100%	100	99.49	99.49	% RSD=0.68
S4:200%	200	200.25	100.12	Mean=100.19%
S5:200%	200	201.02	100.51	S.D=0.291
S6:200%	200	199.89	99.94	% RSD=0.29
S :500%	500	499.23	99.84	Mean = 99.95%
S8:500%	500	500.15	100.03	S.D=0.098
S9 :500%	500	499.91	99.98	% RSD=0.10

TABLE 8: RECOVERY DATA OF CANAGLIFLOZIN				
Sample name	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% Recovery**	**Statistical Analysis**
S1:10%	10	9.98	99.8	Mean=100.03%
S2:10%	10	9.96	99.6	S.D=0.58
S3:10%	10	10.07	100.7	% RSD=0.59
S4:20%	20	19.95	99.75	Mean= 100.38%
S5:20%	20	20.09	100.45	S.D=0.602
S6:20%	20	20.19	100.95	% RSD=0.6
S :50%	50	50.03	100.06	Mean=99.98%
S8:50%	50	49.80	99.6	S.D=0.355
S9 :50%	50	50.15	100.3	% RSD=0.36

Robustness: Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis with respect to deliberate changes in the parameters such as flow rate (1.0 ± 0.2mL), column temperature (30 ± 5 °C), and mobile phase ratio of the mobile phase. The result of robustness study of the developed assay method was established in **Table 9**and **11**. The robustness was established by changing the flow rate, column temperature, and composition of the mobile phase within allowable limits from actual chromatographic conditions. It was observed that there was no marked change in mean RT and RSD is within the limit of ≤2. The tailing factor, resolution factor, and No. of theoretical plates are found to be acceptable limits for both MET and CANA.

TABLE 9: ROBUSTNESS (CHANGE IN FLOW RATE) FOR METFORMIN HYDROCHLORIDE AND CANAGLIFLOZIN

Drug	Flow rate (ml/min)	% Assay	% SD	% RSD
Metformin Hydrochloride	0.8	99.5	1.6	1.6
	1	100.62	1.08	1.1
	1.2	99.4	1.5	1.5
Canagliflozin	0.8	99.4	1.4	1.4
	1	99.53	0.57	0.6
	1.2	100.23	1.3	1.29

TABLE 10: ROBUSTNESS (CHANGE IN COLUMN TEMPARATURE) FOR METFORMIN HYDROCHLORIDE AND CANAGLIFLOZIN

Drug	Change in Column temperature	% Assay	% SD	% RSD
Metformin Hydrochloride	25°C	99.42	1.20	1.2
	30°C	100.28	1.06	1.06
	35°C	100.62	1.08	1.1
Canagliflozin	25°C	100.16	1.21	1.2
	30°C	99.53	0.57	0.6
	35°C	100.81	0.43	0.4

IV. CONCLUSION

"A REVIEW STUDY OF "ANALYTICAL VALIDATION OF STABILITY INDICATING REVERSE PHASE - HPLC FOR METFORMIN HYDROCHLORIDE-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 100-500µg/ml for Metformin Hydrochloride and 10-50µg/ml for Canagliflozin with correlation coefficients (r = 0.999). The percentage recoveries of Metformin Hydrochloride and Canagliflozin were achieved in the range of 99.5-100.2% 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, economic, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust.

V. ACKNOWLEDGEMENT:

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VI. DISCLOSURE STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for content and writing of this article.

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