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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR METFORMIN AND CANAGLIFLOZIN BY RP-HPLC

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ABSTRACT

Bioanalytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a biological matrix. A simple, selective, rapid, precise and economical Reverse-Phase HPLC method has been developed and validated for quantitative determination of Metformin and Canagliflozin. Metformin Hydrochloride is an orally administered biguanide derivative used to lower blood glucose concentration in patients with non insulin dependent diabetes mellitus. Canagliflozin is an anti diabetic drug used to improve glycemic control in patients with type 2 diabetes. Pioglitazone is used as an internal standard. The method was carried out with Waters HPLC with auto sampler and PDA detector. Spursil (Dikma) ODS C $_{18}$ column (4.6 x 250mm, 5µm) is used at a flow rate of 1.0mL/min. Detection was carried out at 254 nm. The mobile phase used is Phosphate buffer (pH 3.0) with Acetonitrile in proportion 85: 15 v/v respectively. The retention times of Metformin and Cangliflozin were 4.738min and 8.352min respectively. The method was developed and tested for linearity range of 250 to 1250 ng/mL for Metformin HCl and 25 to 125 ng/mL for Canagliflozin. These bioanalytical validations play a significant role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies.

In which different parameters like accuracy, precision, selectivity, sensitivity, reproducibility, and stability are performed.

KEYWORDS: Metformin Hydrochloride, Canagliflozin, RP-HPLC Method, Validation.

INTRODUCTION

Methods of measuring drugs in biological media are becoming increasingly important for the study of bioavailability & bioequivalence studies, quantitative evaluation of drugs and their metabolites, new drug development, clinical pharmacokinetics, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring.[1,2,3,4] **METFORMIN** is chemically 1-carbamimidamido-N,N-dimethylmethanimidamide. It decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.[5,6,7]

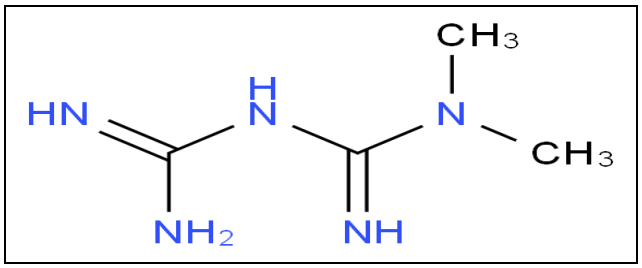

Figure 1: Chemical Structure of Metformin.

CANAGLIFLOZIN is chemically (2S,3R,4R,5S,6R)-2-[3-[[5-(4-fluorophenyl)thiophen-2-yl]methyl]-4-methylphenyl]-6-(hydroxymethyl)oxane-3,4,5-trio.It is a medication used for the treatment of type 2 diabetes It is of the gliflozin class or subtype 2 sodium-glucose transport (SGLT-2) inhibitors class. This mechanism is associated with a low risk of hypoglycaemia (too low blood glucose) compared to sulfonylurea derivatives and insulin. Canagliflozin is an inhibitor of subtype 2 sodium-glucose transport proteins (SGLT2), which is responsible for at least 90% of renal glucose reabsorption (SGLT1 being responsible for the remaining 10%. Blocking this transporter causes up to 119 grams of blood glucose per day to be eliminated through the urine.[8,9,10] **PLIOGLITAZONE** is used as internal standard.

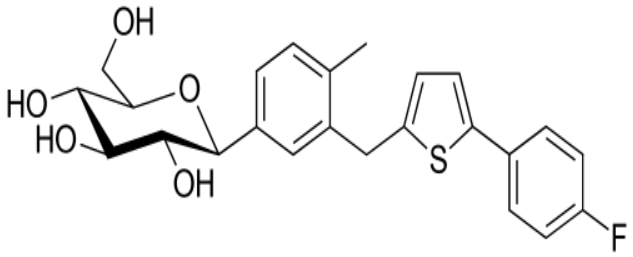

Figure 2: Chemical Structure of Canagliflozin.

Literature survey revealed that a validated HPLC method has been developed for the estimation of the metformin and canglifloxin.[11] formulation but not in the biological fluid so attempt was made to develop a new HPLC method with combination of metformin and canaglifloxin in rat plasma. This method is considered to be more suitable since this is a powerful and rugged method. It is also extremely specific, linear, precise, accurate, sensitive and rapid. In this study we have developed HPLC method with a protein precipitation extraction and improved sensitivity for the determination of Metformin and Canaglifloxin in rat plasma and the developed method is validated as per regulatory requirements.

MATERIALS AND METHODS

Chemicals

Metformin, Canaglifloxin and Plioglitazone was gifted by HIQ Pharma Labs. HPLC Grade solvents (Acetonitrile, Methanol) and Ortho phosphoric Acid were obtained from MERCK and milli-Q water was from SG Series Compact Pretreatment Module. AR Grade Potassium dihydrogen phosphate was purchased from FINER chemical LTD.

Instrument

WATERS 2695 HPLC with software Empower separation module with a UV detector and autosampler. Chromatographic separations were performed using Spursil (Dikma) ODS C_{18} (4.6 x 250mm, 5µm) column. Ultrasonic bath (Toshcon by Toshniwal), digital Ph meter (Systronics model 802) were used in the study.

Preparation of Metfromin And Canagliflozin Standard Stock Solution

Accurately weigh 250mg of Metformin, 25mg of Canagliflozin working standard and it is transfer into a 10 ml of volumetric flasks, to this add 7 ml of diluents vortex for 10 mins. (stock -1). From the Above stock solution take 1 ml and make upto 10 ml with diluents. (stock-2). From the STOCK-2 taken 100 µl and make upto 10 ml with the diluents. (stock -3).

Preparation of internal standard (pioglitazone)

Accurately weigh 100 mg of Pioglitazone working standard and it is transfer into a 10 ml of volumetric flasks, to this add 7 ml of diluents vortex for 10 mins. (stock -1). From the Above stock solution take 1 ml and make upto 10 ml with diluents. (stock-2). From the STOCK-2 taken 100µl and make upto 10 ml with the diluents. (stock -3).

Optimized extraction procedure

After taking trials with different methods of extraction, protein precipitation method was optimized with 2% v/v acetic acid in acetonitrile as a precipitating agent for the extraction of metformin and Canagliflozin from the plasma and following procedure was used.

0.1 mL (100 microlitre) of validation/study sample was taken in a micro centrifuge tube and 0.300 mL(300 microlitre) of IS working solution was added except in blank plasma, where 0.10 mL of diluent was added in place of IS working solution. 0.01 mL of 50 mM sodium hydroxide was added and mixed on the vortexer. To that mixture, 0.75 mL of 2% acetic acid in acetonitrile was added and centrifuged for 10 min at 10,000 r.p.m. at 4^0C. 0.2 mL of supernatant was transferred in to auto sampler vial and 0.2 mL of reconstitution solution was added and injected in to HPLC system.[12]

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Instrument used	:	Waters HPLC with auto sampler and PDA detector.
Temperature	:	Ambient
Column	:	SPURSIL ODS C_{18} (4.6 x 150mm, 3µm)
Buffer	:	3.4g potassium dihydrogen ortho phosphate was taken in a 1000ml volumetric flask and adjust the pH with Diluted OPA upto 3.
Mobile phase	:	85% buffer 15% Acetonitrile
Flow rate	:	1 ml per min
Wavelength	:	254 nm
Injection volume	:	20µl
Run time	:	15 min.

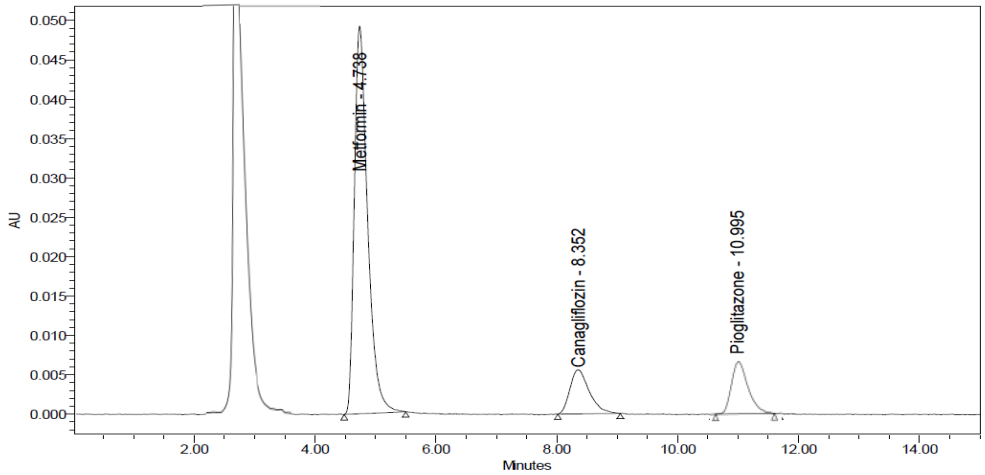

Figure.3: Typical Chromatogram of Metformin And Canaglifloxin.

BIOANALYTICAL METHOD VALIDATION

Calibration curve

Calibration curve consisted of reconstitution solution, blank sample (matrix sample processed without internal standard and analyte), zero sample (matrix sample processed with internal standard) and non-zero samples (calibration standards). The calibration curves were linear over the range of 250 to 1250 ng/mL for Metformin HCl and 25 to 125 ng/mL for Canagliflozin. The coefficient of determination was ≥0.9990.

Table 1: Calibration data of metformin.

S.NO	CONCENTRATION (ng/ml)	Peak area
1	250	23645
2	500	47283
3	750	69365
4	1000	91536
5	1250	115283
		Y= 91.01+1163 R^2 = 0.999

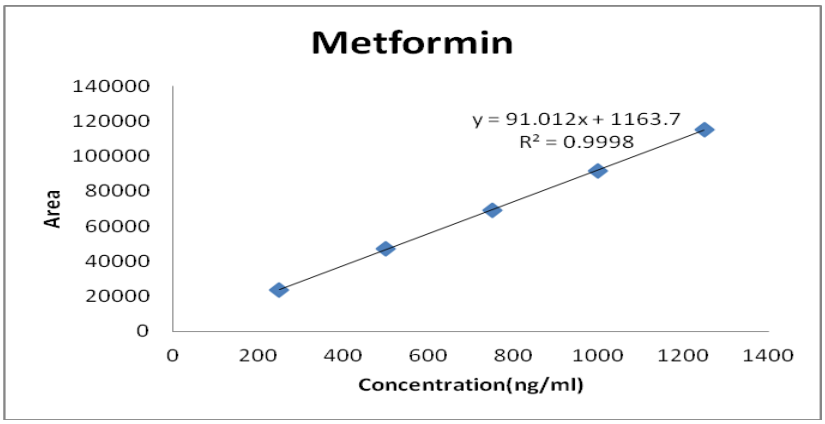

Figure 4: Linearity Graph of Metformin.

Table 2: Calibration data of canagliflozin.

S.NO	CONCENTRATION (ng/ml)	Peak area
1	25	5243
2	50	10553
3	75	15953
4	100	21587
5	125	27253
		y= 220.0x-398.4 R^2 = 0.999

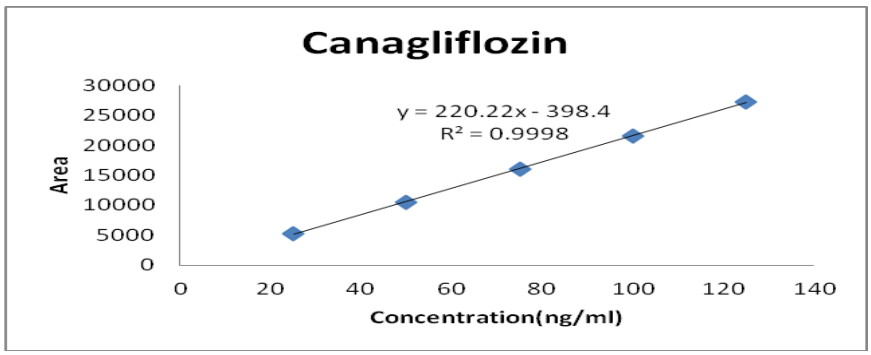

Figure 5: Linearity Graph of Canaglifloxin.

Specificity/Selectivity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. For selectivity, blank plasma of two different lots were taken and analyzed. Selectivity was evaluated by comparing extracted blank plasma response with extracted LLOQ. No significant interference from the blank plasma was observed at the retention times of both analyte and internal standards in both drugs.

Sensitivity

This was performed by injecting six different aliquots of extracted LLOQ concentration. Percentage deviation from the nominal concentration and percentage CV were calculated. The developed methods were found to be sensitive with % RSD.

Carryover

Carry over test was performed to confirm the absence carryover of analyte from the previous injection. The experiment was performed at ULOQ level. The samples were injected in the following order: blank reconstitution solution; aqueous equivalent ULOQ sample; blank reconstitution solution; aqueous equivalent ULOQ sample; blank reconstitution solution; extracted blank matrix sample; extracted ULOQ sample; extracted blank matrix sample; extracted ULOQ sample; extracted blank matrix sample, followed by three LLOQs. No significant carryover was observed in both CAN and MET studies.

Accuracy and Precision

Within-batch accuracy and precision were assessed by analyzing one calibration curve and 2 sets of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) in different batches. Between batch accuracy and precision evaluation were also assessed by analyzing 2 batches on different days. The accuracy and precision for all the batches at LLQC and LQC, MQC and HQC levels were calculated. Mean percentage nominal concentration and coefficient of variation for all the batches individually (intra) and collectively (inter) were found to be within acceptance limits for both CAN and MET studies.

Table 3: Results Showing Precision for Metformin, Canaglifloxina and Pliоglitazone.

S.NO	METFORMIN	CANAGLIFLOZIN	PIOGLITAZONE
1	68365	15634	12692
2	68653	15675	12756
3	67345	15967	12667
4	68674	15637	12534
5	67567	15835	12645
6	67863	15785	12853
AVERAGE	68077.8	15755.5	12691.2
MEAN	568.0	132.1	107.5
% RSD	0.8	0.8	0.8
	68077.8	15755.5	12691.2

RECOVERY

Post spiked and extracted quality control samples were analyzed and percentage recovery at each level was calculated by comparing the response area of low, medium and high quality control levels and an internal standard. Mean percentage recovery across all QC levels for CAN and MET is 99.45% and 99.26% respectively.

Table 4: Results Showing Recovery for Metformin.

		Peak Area	Amount Added	Amount Found	Recovery	Mean Recovery
METFORMIN	LQC	33948	125	124.36*	99.49	99.95
		33886				
		33967.				
	MQC	68352	250	250.90*	100.36	
		68486.				
		68542				
	HQC	102427	375	374.95*	99.99	
		102242				
		102256				

*n=3 replicates mean area found

Table 5: Results Showing Recovery for Canaglifloxin.

		Peak Area	Amount Added	Amount Found	Recovery	Mean Recovery
CANAGLIFLOZIN	LQC	7836	12.5	12.38*	99.08	99.77
		7763				
		7863				
	MQC	15835	25	25.03*	100.11	
		15736				
		15842				
	HQC	23547	37.5	37.55*	100.14	
		23856				
		23735				

Stock Solution Stability

Both, main stock and spiking stocks of Canaglifloxin and Metormin studies were found to be stable at 2-8^{o}C for 9 days (long term) and for 8 hours at room temperature.

Table 6: Results Showing Stock Solution Stability for Metformin.

S.NO	METFORMIN	CANAGLIFLOZIN	PIOGLITAZONE
1	67326	15276	11937
2	67189	15234	12048
3	67593	15297	11942
AVERAGE	67369.3	15269.0	11975.7
MEAN	205.5	32.1	62.7
% RSD	0.3	0.2	0.5

Bench Top Stability

Samples were prepared at low and high quality control levels and kept at bench top at room temperature for a minimum of four hours (stability samples). Later, fresh calibration standards and quality control samples (comparison samples) were prepared, extracted and analyzed with the stability samples. Mean percentage change was calculated.

Table 7: Results Showing *Bench Top Stability* for Metformin, Canaglifloxin and Plioglitazone.

S.NO	METFORMIN	CANAGLIFLOZIN	PIOGLITAZONE
1	66536	15436	12534
2	66752	15552	12564
3	66863	15455	12646
AVERAGE	66717.0	15481.0	12581.3
MEAN	166.3	62.2	58.0
% RSD	0.2	0.4	0.5

Freeze and Thaw Stability

The samples were exposed to four freeze thaw cycles. Stability samples were analyzed against fresh calibration standards and quality control samples (comparison samples). Mean % change was calculated and verified against acceptance criteria. The plasma samples were found to be stable after repeating three cycles of freezing at -20oC and thawing to room temperature.

Table 8: Results Showing Freeze and Thaw Stability for Metformin, Canaglifloxin And Plioglitazone.

S. No	Metformin	Canagliflozin	Pioglitazone
1	65833	15062	12035
2	65936	15175	12056
3	65526	15201	12071
AVERAGE	65765.0	15146.0	12054.0
MEAN	213.3	73.9	18.1
% RSD	0.3	0.5	0.2

In-Injector Stability

Stability of processed samples in the instrument over the anticipated run time was assessed. Stability samples were analyzed with fresh calibration standards along with low and high quality control samples (comparison samples) and mean percentage change was calculated. The plasma samples were found to be stable 4^{O}C for 30 hours with mean% change.

Table 9: Results Showing In-Injector Stability for Metformin, Canaglifloxin and Plioglitazone.

S.no	Metformin	Canagliflozin	Pioglitazone
1	66635	15072	12037
2	66826	15184	12142
3	66576	15112	12064
AVERAGE	66679.0	15122.7	12081.0
MEAN	130.7	56.8	54.5
% RSD	0.2	0.4	0.5

Stability

The stability of the analytes in plasma were assessed at different conditions anticipated during the pharmacokinetic studies like bench top stability, freeze thaw stability, in-injector stability and long term stability at LQC and HQC levels. The results are summarized below.

Table 10: Results Showing Stability for Metformin, Canaglifloxin and Plioglitazone.

S.NO	STABILITY	% DEGRADATION METFORMIN	% DEGRADATION CANAGLIFLOZIN	% DEGRADATION PIOGLITAZONE
1	Freez Thraw stability	3.4	3.9	5.02
2	Benchtop stability	2	1.74	0.87
3	IN injector stability	2.05	4.02	4.81
4	Solution stability	1.04	3.09	5.64

CONCLUSION

The current validated HPLC method for Canagliflozin and Metformin offers good accuracy and significant advantages in terms of sensitivity, selectivity and sample preparation. It can be used for the estimation of Canagliflozin and Metformin in biofluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak without any interference From the results we conclude that the developed method can be applied in bioequivalence, pharmacokinetic and toxicokinetic studies with desired precision and accuracy along with high-throughput.

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