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A RP-HPLC Method Development and Validation of Metformin and Dipagliflozin in Bulk and Dosage Form

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ABSTRACT

The estimation of Metformin and Dapagliflozin was done by RP-HPLC. The assay of Metformin and Dapagliflozin was performed with tablets and the % assay was found to be 99.81 and 100.44 which shows that the method is useful for routine analysis. The linearity of Metformin and Dapagliflozin was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The method shows precision 0.5 and 0.3 for Metformin and Dapagliflozin which shows that the method is precise. The method shows precision 0.5 and 0.3 for Metformin and Dapagliflozin which shows that the method is repeatable when performed in different days also. The total recovery was found to be 99.1% and 99.99% for Metformin and Dapagliflozin. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The LOD and LOQ for Metformin was found to be 3 and 9.8 and LOD and LOQ for Dapagliflozin was found to be 2.98 and 10. The robustness limit for mobile phase variation and flow rate variation is well within the limit, which shows that the method is having good system suitability and precision under a given set of conditions. The degradation results are also in limits.

INTRODUCTION

Metformin (1-carbamimidamido-N, N-dimethyl methanimidamide) is a biguanide antihyperglycemic agent used for treating non-insulin-dependent diabetes mellitus (NIDDM). It is soluble in Water and Ethanol with melting point 223-226^{o}C . It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption and increasing insulin-mediated glucose uptake. Metformin is the only oral antihyperglycemic agent that is not associated with weight gain. Metformin may induce weight loss and is the drug of choice for obese NIDDM patients. Its main side effects are dyspepsia, nausea, and diarrhea. Dose titration and/or use of smaller divided doses may decrease side effects. Metformin 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.

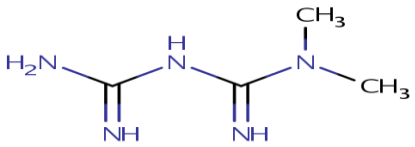

Fig 1: Metformin

Dapagliflozin(2S,3R,4R,5S,6R)-2-{4-chloro-3-[(4-methoxyphenyl)methyl]phenyl}-6-(hydroxymethyl)oxane-3,4,5-triol is indicated for the management of diabetes mellitus type 2. It is a sodium-glucose cotransporter 2 inhibitor, which prevents glucose reabsorption in the kidney. Using dapagliflozin leads to heavy glycosuria (glucose excretion in the urine), which can lead to weight loss and tiredness. Dapagliflozin was approved by the FDA on Jan 08, 2014. Dapagliflozin is not recommended for patients with type 1 diabetes mellitus or the treatment of diabetic ketoacidosis.

Structure:

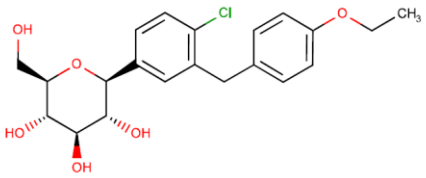

Fig 2: Dapagliflozin

Dapagliflozin is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide, which should be purged with an inert gas. The solubility of dapagliflozin in these solvents is approximately 30 mg/ml.

MATERIALS AND METHODS

Instruments used:

The liquid chromatographic systems used were HPLC WATERS, software: Empower, 2695 separation module, PDA detector, LABINDIA UV 3000+ UV/VIS spectrophotometer, Adwa – AD 1020 pH meter, Afcoset ER-200A Weighing machine, Borosil Pipettes, Burettes, Beakers.

Chemicals used:

The gift samples of Metformin and Dapagliflozin were procured from PHARMATRAIN, KH2PO4 was purchased from FINER chemical LTD, Water and Methanol for HPLC LICHROSOLV (MERCK), Acetonitrile for HPLC was purchased from MOLYCHEM and Ortho phosphoric Acid from MERCK.

Method development:

Standard Solution Preparation:

Accurately weigh and transfer 500 mg of Metformin and 5 mg of Dapagliflozin working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 500 mg of Metformin and 5 mg of Dapagliflozin tablet powder into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of Phosphate buffer:

3.4g of KH_2PO_4 in 1000 ml of HPLC water Ph was adjusted with 0.1M NAOH up to 3.0. Final solution was filtered through 0.45 µm Membrane filter and sonicated it for 10 mins.

Preparation of mobile phase:

Accurately measured 500 ml (50%) of the above buffer and 500 ml of Acetonitrile HPLC (50%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Mobile Phase Optimization:

Initially, the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to 0.025M Phosphate buffer (pH 3.0) and acetonitrile in proportion 50: 50 v/v respectively.

Wavelength selection:

UV spectrum of 10µg/ml Metformin and 10µg/ml Dapagliflozin in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength was selected as 240nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like the C18 column Phenomenex column, YMC, and Inertsil ODS column. Inertsil ODS column C8 (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

In this optimized chromatographic analysis, a Waters HPLC system equipped with an auto-sampler and UV detector was employed to separate and detect compounds. The analysis was conducted at ambient temperature using an Inertsil ODS C8 column, which has dimensions of 4.6 mm inner diameter, 250 mm length, and 5-micrometer particle size. The mobile phase consisted of a 50% phosphate buffer with a pH of 3.0 and 50% acetonitrile. A flow rate of 1 mL per minute was maintained to facilitate the elution of compounds. Detection was performed at a wavelength of 240 nm using the UV detector. Samples were injected at a volume of 20 microliters, and the entire chromatographic run was completed in 8 minutes. These specific conditions were meticulously chosen to ensure accurate and efficient separation of the target compounds in the sample.

Method validation:

System suitability: The tailing factor for the peaks due to Metformin and Dapagliflozin in Standard solution should not be more than 2.0. Theoretical plates for the Metformin and Dapagliflozin peaks in Standard solution should not be less than 2000.Resolution for the Metformin and Dapagliflozin peaks in standard solution should not be less than 2.

The standard and sample solutions were injected five times and peak areas of injections were measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The results were given in Table 2.

Specificity:

For Specificity Blank and Standard are injected into system. There is no any interference of any peak in the blank with the retention time of the analytical peaks.

ASSAY:

Three replicates injections of standard and sample solutions were injected and the assay was calculated by using formula and the results were given in table-3.

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Precision:

The standard solution was injected for six times and the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. The results are summarized for Metformin and Dapagliflozin in table -4.

Acceptance Criteria:

The % RSD for the area of six standard injection results should not be more than 2%**.**

Intermediate precision/ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day. The standard solutions prepared in the precision was injected on the other day, for six times and the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. The results are summarized for Metformin and Dapagliflozin in table-5.

Acceptance Criteria:

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

Accuracy:

The accuracy of the newly developed method was evaluated by recovery studies at three different levels equivalent to 50,100 & 150%. At each level the target concentration was spiked in triplicates and the amount recovered was calculated the percentage recovery at each level was calculated and reported in table-6 and 7 for metformin and dapagliflozin.

Acceptance Criteria:

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

Linearity:

A Series of solutions were prepared using favipiravir working standard at concentration levels from 500ppm to 2500 ppm of metformin and 5ppm to 25 ppm of Dapagliflozin of target concentration. Each sample solution was injected into the HPLC system in replicates

and the peak areas were measured. A graph was plotted with peak areas vs concentrations and the r2 value was calculated. The results are shown in table – 8.

Limit of detection and validation:

From the linearity data the limit of detection and quantification were calculated using the following formulae respectively and results were reported in table -9.

$$LOD = \frac{3.3\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

$$LOQ = \frac{10\ \sigma}{S}$$

The values are given in table

Robustness:

A study was conducted to determine the effect of variation in flow rate, change in mobile phase composition and detection of wavelength. A standard solution prepared as per the test method was injected into the HPLC system using flow rates, 0.9ml/min to 1.1ml/min. The same studies were also performed by varying mobile phase composition and detection wavelength. The system suitability parameters were evaluated and reported in table 9, 10,11,12.

Degradation:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. This work aimed to perform the stress degradation studies on Metformin and Dapagliflozin using the proposed method and results were reported in table 13.

Preparation of stock:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 500 mg of Metformin and 5 mg Dapagliflozin in sample into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make

volume up to the mark with the same solvent. Then it is Filtered through 0.44 micron Injection filter. (Stock solution).

Hydrolytic degradation under acidic condition

Pipette 0.3 ml of above solution into a 10-volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 micron syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.3 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation

Metformin and Dapagliflozine samples were taken in a petri dish and kept in Hot air oven at 110^0 C fo 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and 1ml of 30% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:

Pipette 0.3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 micron syringe filters and place in vials.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT:

Optimized Chromatogram (Standard)

Mobile phase ratio : Methanol: Acetate Buffer (pH-4.2) (40:60 v/v)

Column : Inertsil ODS C18 (4.6mm x 250mm, 5µm)

Column temperature : 35ºC

Wavelength : 323nm

Flow rate : 1.0ml/min

Injection volume : 10µL

Run time : 10min

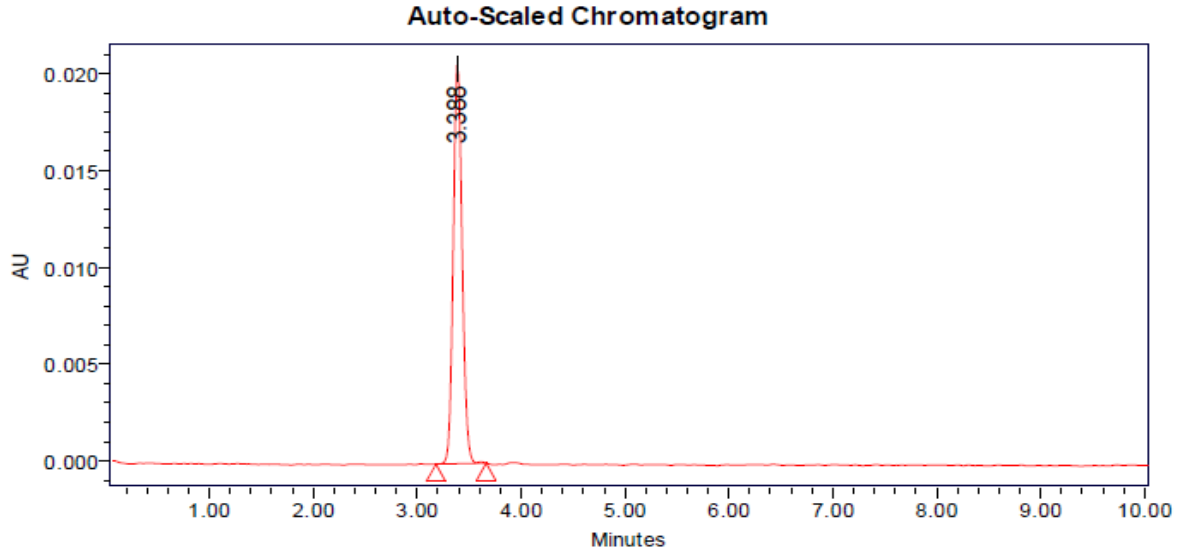

Fig 3: Optimized Chromatogram (Standard)

Table 1: Data of optimized chromatogram

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Favipiravir	3.388	145867	32546	1.76	8457

METHOD VALIDATION:

System suitability:

The theoretical plates are more than 2000 and the tailing factor is less than 2 in each injection for both the analytes. The values were within the acceptance criteria.

Table 2: Results of System Suitability for Favipiravir

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Favipiravir	3.398	145965	32653	8475	1.78
2	Favipiravir	3.324	146857	32785	8495	1.79
3	Favipiravir	3.349	145985	32598	8492	1.80
4	Favipiravir	3.388	146697	32695	8463	1.76
5	Favipiravir	3.364	145982	32675	8458	1.77
Mean			146380.25			
Std. Dev.			462.762			
% RSD			0.316137			

ASSAY:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.

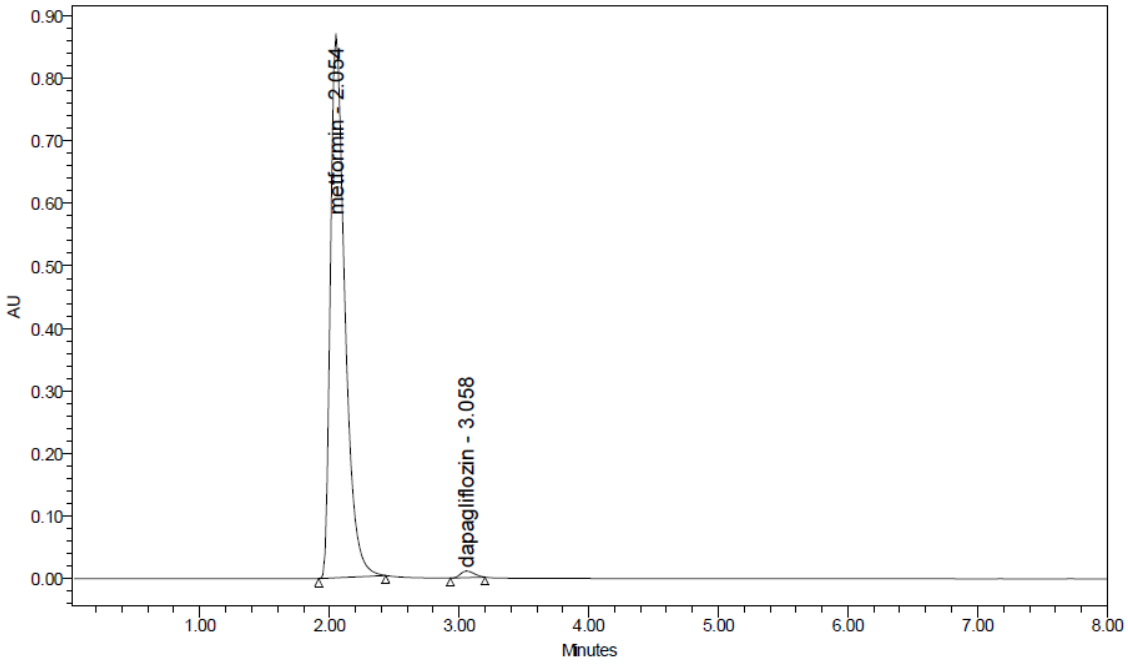

Figure 4: Chromatogram for Standard

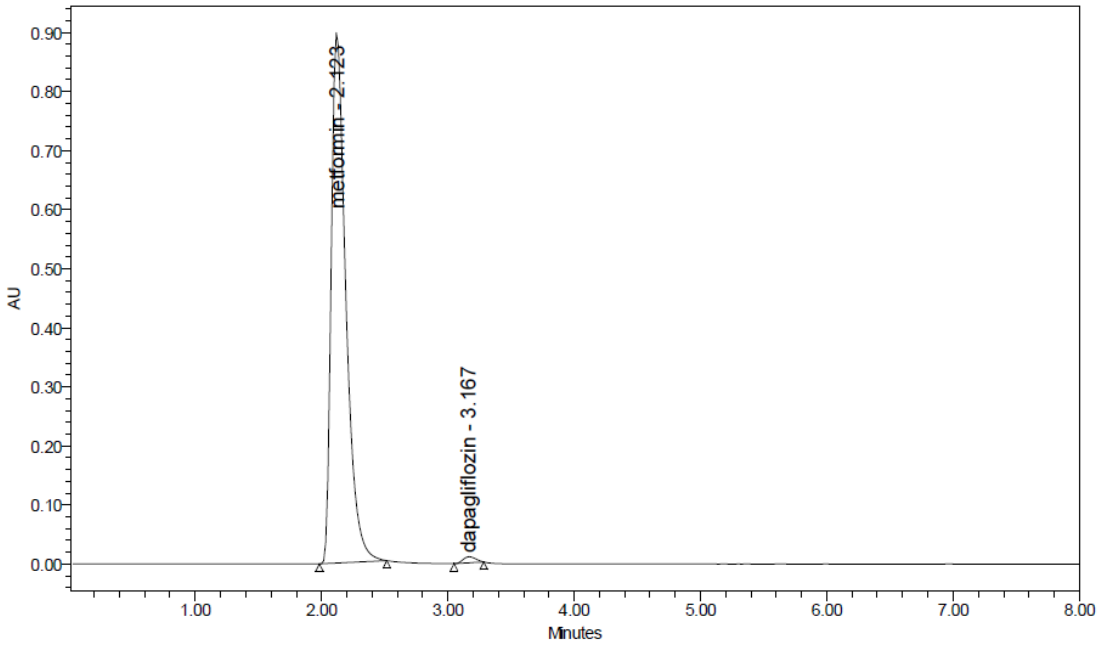

Figure 5: Chromatogram for Sample

Table 3: Results of Assay for Metformin and Dapagliflozin

	Label Claim (mg)	**% Assay**
Metformin	500	99.81
Dapagliflozin	5	100.44

Table 4: Results of Precision for Metformin and Dapagliflozin

Injection	Area for Metformin	Area for Dapagliflozin
Injection-1	8311797	83614
Injection-2	8396358	83101
Injection-3	8310349	83575
Injection-4	8341898	83737
Injection-5	8395391	83249
Injection-6	8382972	83812
Average	8356491.2	83514.7
Standard Deviation	40316.7	280.4
%RSD	0.5	0.3

Acceptance criteria:

- %RSD for the sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence the method is precise.

INTERMEDIATE PRECISION (ruggedness)

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day-to-day and system to system variation.

Table 5: Results of Intermediate Precision for Metformin & Dapagliflozin

Injection	Area for Metformin	Area for Dapagliflozin
Injection-1	8310627	83945
Injection-2	8362992	83191
Injection-3	8396358	83728
Injection-4	8307422	83705
Injection-5	8360678	83792
Injection-6	8389407	83337
Average	8354580.7	83616.3
Standard Deviation	38005.3	289.2
%RSD	0.5	0.3

Acceptance criteria:

- %RSD of five different sample solutions should not be more than 2.
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Table 6: Accuracy (recovery) data for Metformin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	4157603	250	249.04	99.62	99.91
100%	8318303	500	498.27	99.65	
150%	12578593	750	753.46	100.46	

*Average of three determinations

Table 7: Accuracy (recovery) data for Dapagliflozin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	41536	6.25	6.22	99.49	99.99
100%	83577	12.5	12.51	100.10	
150%	125727	18.75	18.82	100.38	

*Average of three determinations

Acceptance Criteria:

- The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, and 150% are within the limits. Hence method is accurate.

LINEARITY:

The linearity range was found to lie from 500µg/ml to 2500µg/ml of Metformin, 5µg/ml to 25µg/ml 0f Dapagliflozin and chromatograms are shown below.

Table 8: Area of different concentrations of Metformin and Dapagliflozin

S. No	Metformin		Dapagliflozin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	500	1813801	5	35108
2	1000	4832300	10	60347
3	1500	8319014	15	83408
4	2000	11957810	20	110257
5	2500	15400509	25	132422

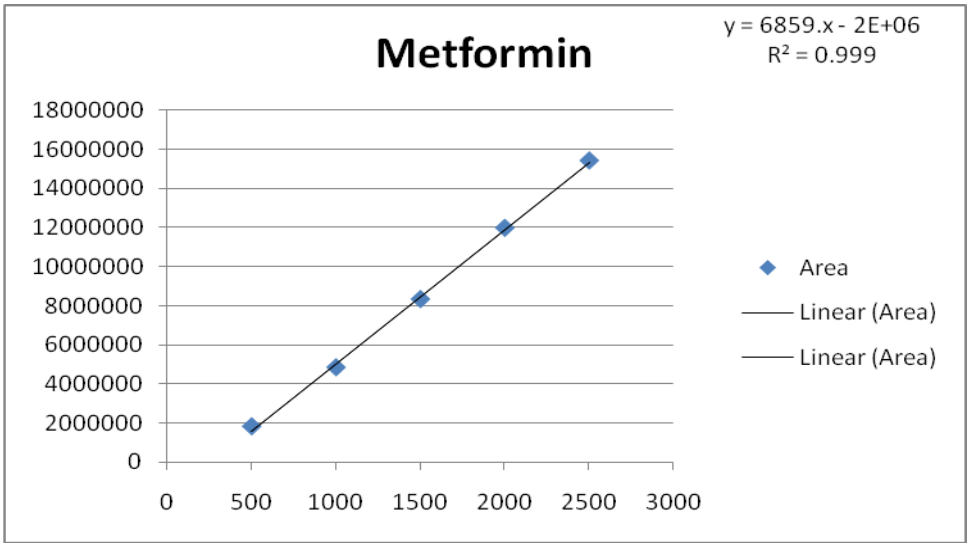

Fig 6: Calibration graph for Metformin

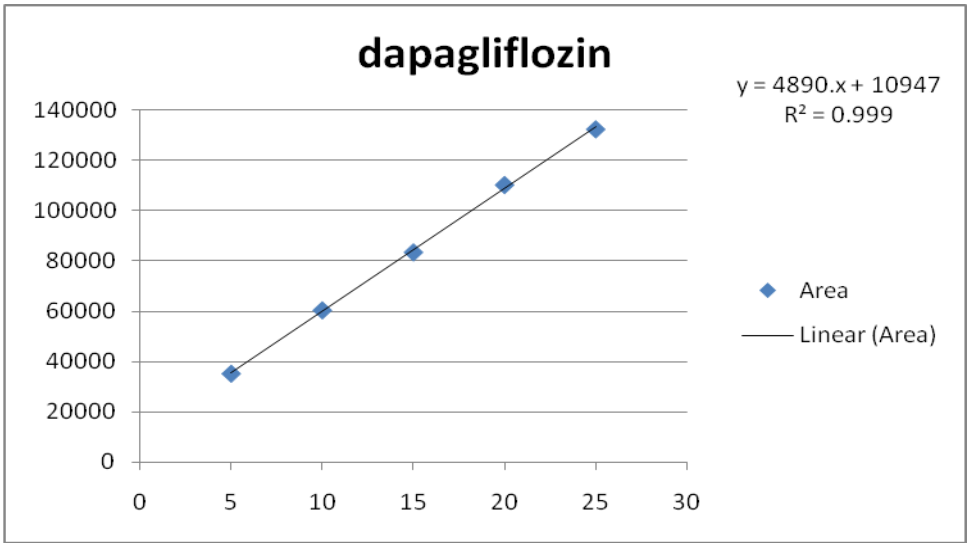

Fig 7: Calibration graph for Dapagliflozin

Acceptance criteria:

The correlation coefficient (R^2) should not be less than 0.999.

- The correlation coefficient obtained was 0.999 which is in the acceptance limit.

LIMIT OF DETECTION FOR METFORMIN AND DAPAGLIFLOZIN

The lowest concentration of the sample was prepared concerning the base-line noise and measured the signal to noise ratio and measured the signal to noise ratio.

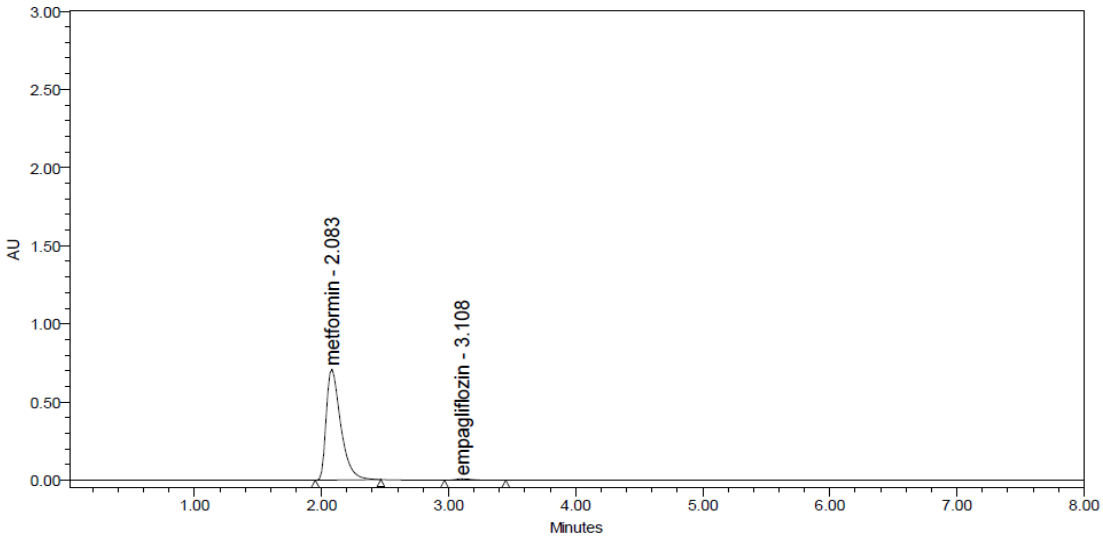

Fig 8: Chromatogram of Metformin, Dapagliflozin showing LOD

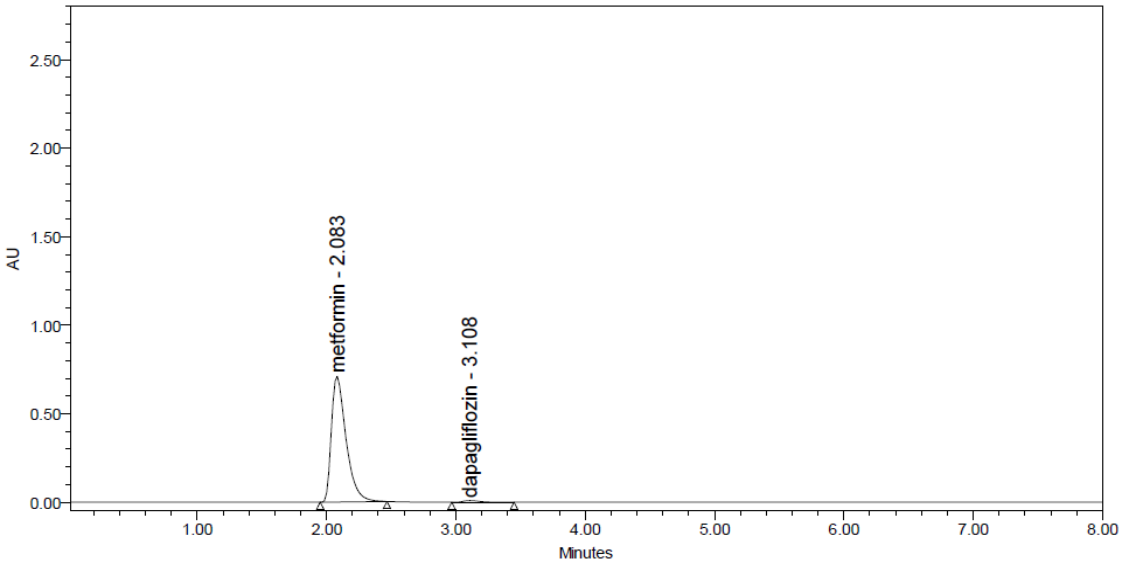

Figure 9: Chromatogram of Metformin, Dapagliflozin showing LOQ

ROBUSTNESS:

The standard and samples of Metformin and Dapagliflozin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 9 Results for variation in flow for Metformin

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2828.94	3.41
2	1.0	262771	1.52
3	1.1	2576.94	1.32

Table 10: Results for variation in flow for Dapagliflozin

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	2528.32	1.42	6.61
2	1.0	2625.13	4.94	1.17
3	1.1	2878.32	1.25	9.61

***** Results for actual flow (1.0ml/min) have been considered from the Assay standard.

Table 11: Results for variation in mobile phase composition for Metformin

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2728.94	1.56
2	*Actual	2627.71	1.55
3	10% more	2628.94	1.42

Table 12: Results for variation in mobile phase composition for Dapagliflozin

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	2928.32	1.35	6.5
2	*Actual	2625.13	1.17	4.94
3	10% more	3269	1.35	9.61

***** Results for actual Mobile phase composition have been considered from the Accuracy standard.

Acceptance criteria:

The Retention time, USP plate count, USP tailing factor obtained for change of flow rate, and variation in the mobile phase was found to be within the acceptance criteria. Hence the method is robust.

Table 13: Degradation Results for Metformin and Dapagliflozin

Sample Name	Metformin		Dapagliflozin	
	Area	% Degraded	Area	% Degraded
Standard	8330541		83329.8	
Acid	7978259	4.23	78259	6.09
Base	7379321	11.42	79321	4.81
Peroxide	7704978	7.51	74978	10.02
Thermal	7714851	7.39	74851	10.17
Photo	7914789	4.99	74789	10.25

SUMMARY AND CONCLUSION

The estimation of Metformin and Dapagliflozin was done by RP-HPLC. The assay of Metformin and Dapagliflozin was performed with tablets and the % assay was found to be 99.81 and 100.44 which shows that the method is useful for routine analysis. The linearity of Metformin and Dapagliflozin was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should not be more than 2.0% and the method shows precision 0.5 and 0.3 for Metformin and Dapagliflozin which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should not be more than 2.0% and the method show precision of 0.5 and 0.3 for Metformin and Dapagliflozin which shows that the method is repeatable when performed on different days also. The accuracy limit is the percentage recovery should be in the range of 98.0% - 102.0%. The total recovery was found to be 99.1% and 99.99% for Metformin and Dapagliflozin. The validation of the developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10. The LOD and LOQ for Metformin was found to be 3 and 9.8 and LOD and LOQ for Dapagliflozin was found to be 2.98 and 10.

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