Available Online at

http://www.ijcpa.in

January-March 2020

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726; pISSN: 2395-2466

DOI: http://dx.doi.org/10.21276/ijcpa

Research Article | Volume-7 | Issue-2 | Article ID: 0031

AN IMPROVED VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN AND DAPAGLIFLOZIN

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Received: 19 April 2019 / Revised: 16 December 2019 / Accepted: 2 January 2020 / Available online 31 March 2020

ABSTRACT

Objective: A simple and precise reversed-phase high-performance liquid chromatography method was developed and validated for the simultaneous determination of metformin (MET) hydrochloride and dapagliflozin (DAP) in bulk and pharmaceutical dosage form. Methods: Chromatography was carried out on Unisol C18 (150 mm × 4.6 mm, 3 µ particle size) column containing mobile phase of Methanol: Buffer (Triethanolamine adjusted to pH 3.5 with orthophosphoric acid in the ratio of 80:20 %/v/v) at a flow rate of 0.8 ml/minute. The analyte was monitored using photodiode array detector at 245 nm. Results: The retention time was found to be 2.040 minutes and 3.733 minutes for DAP and MET hydrochloride, respectively. The proposed method was found to be having linearity in the concentration range of 20-120 µg/ml for MET (R^2= 0.9988) and 10-60 µg/ml for DAP (R^2= 0.9989), respectively. The mean % recoveries obtained were found to be 99.47-100.21 % for MET and 98.2-99.9 % for DAP respectively. The method developed has been statistically validated according to ICH guidelines. Conclusion: Hence the optimized method can be successfully applied for the simultaneous determination of Metformin hydrochloride and Dapagliflozin in the routine quality control analysis.

Keywords – *Dapagliflozin, Metformin hydrochloride, RP HPLC, Method validation.*

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic progressive metabolic disorder characterized by insulin deficiency. Pancreatic β-cell function is gradually deteriorated in patients of Type 2 DM which is reflected into inadequate glycemic control on a long run. Expected rise in prevalence of diabetes is mainly due to increased life span because of better healthcare facilities and increase in diabetic risk factors, especially physical inactivity and obesity due to sedentary lifestyle [1].

1.1. Drug profile

1.1.1 Dapagliflozin

Dapagliflozin inhibits subtype 2 of the sodium-glucose transport proteins (SGLT2) which are responsible for at least 90 % of the glucose reabsorption in the kidney. Blocking this transporter mechanism causes blood glucose to be eliminated through the urine.

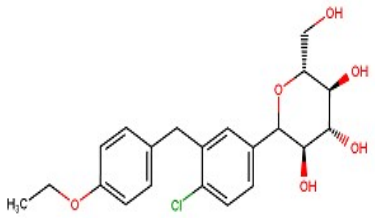

Figure-1: Chemical structure of Dapagliflozin

Dapagliflozin is chemically (2S, 3R, 4R, 5S, 6R)-2-{4- chloro-3-[(4- ethoxyphenyl) methyl] phenyl}-6-(hydroxymethyl [1-4].) oxane-3, 4, 5-triol. Dapagliflozin is a drug of the gliflozin class and it can be used to treat T2DM. Its molecular formula is $C_{21}H_{25}ClO_6$ and molecular weight is 408.873 g/mol (pH - 6.9).

Dapagliflozin is freely soluble in water and very soluble in organic solvents such as methanol, dichloromethane, DMSO and dimethyl formamide.

1.1.2 Metformin

Metformin hydrochloride is an orally administered biguanide derivative used to lower blood glucose concentration in patients with noninsulin dependent diabetes mellitus. Metformin hydrochloride improves insulin sensitivity and decreases insulin resistance by inhibiting complex of the mitochondrial respiratory chain and inducing AMP activated protein kinase dependent signaling.

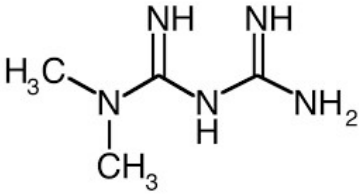

Fig. 2: Chemical structure of Metformin

Metformin hydrochloride (pKa- 12.4) is chemically known as 1, 1-dimethyl biguanide mono hydrochloride. Metformin is a biguanide. The chemical classification of metformin is biguanides. Metformin is primarily used for the treatment of type 2 diabetes that can be used alone or in combination with sulfonylureas, thiazolidinediones or other hypoglycemic agents. It is freely soluble in water, very soluble in organic solvents like methanol and acetonitrile (CAN), practically insoluble in acetone and methylene chloride [1-11].

2. MATERIALS AND METHODS

2.1 Chemicals

The solvents used in this work were HPLC grade methanol, HPLC grade acetonitrile, HPLC grade water procured from Merck India Pvt.Ltd., pH 3.5 buffer adjusted with triethanolamine (pH 8.0) and orthophosphoric acid (1%) which were procured from Qualigen Pvt.Ltd., dapaglifozin and metformin APIs are gifted samples from Aurobindo and the 0.45 µm filter for vacuum filtration was procured from Coastal enterprisers Pvt. Ltd., India.

2.2 Equipment

The instruments used in the study were electronic balance (Mettler toledo), sonicator (PCi analytics, 3.5 L), hot air oven (Kemi), and digital pH meter (Elica). HPLC (Agilent 1200 series) was monitored and integrated using openlab software.

2.3 Chromatographic conditions

HPLC system we chosen was Agilent 1290 series which has an automated rheodyne injector and a PDA detector. All the parameters were selected on the basis of different trials performed and through literature survey and were optimized to yield the best possible results. The reported analytical parameters were selected after evaluating different conditions which may affect results of HPLC analysis, viz. column, ratio of aqueous and organic components of mobile phase, detection wavelength, diluents, concentration of analytes, etc.

The Unisol C18 (150 mm × 4.6 mm, 3µ) was selected owing its advantages of high resolution and reproducibility with relatively low-back pressure and tailing. For the selection mobile phase, initial trials were performed using mobile phases of different composition containing water adjusted to acidic pH (1.8 - 4.0) through the addition of o-phosphoric acid and methanol, 0.1% o-phosphoric acid with methanol, 0.1% formic acid in combination with methanol, etc. However, all these trials resulted in poor peak shapes. Ultimately methanol and triethanolamine buffer of pH 3.5 that offered improved and acceptable peak shape. After selection, the proportion of mobile phase components was optimized to reduce retention times and for better peak shape.

To decide the detection wavelength, the standard solution was scanned over the range of 190-350 nm using PDA detector. Detection wavelength of 245 nm was selected, as it provides results with good response and linearity. This newly developed method was validated as per ICH guidelines. This furnishes evidence that the method is appropriate for its intended use.The optimized chromatographic conditions are shown in **Table 1**.

Table 1: Optimized chromatographic conditions

Sr. No.	Parameters	Chromatographic conditions
1.	Column	Unisol C_{18} column with dimensions (150×4.6 mm) with particle size of 3.0 µm.
2.	Mobile phase	Methanol: Buffer (TEA buffer adjusted to pH 3.5 with 1% OPA) in 80 :20 proportion.
3.	Flow rate	0.8 mL/minute
4.	Injection volume	10 µL
5.	Detector wavelength	245 nm
6.	Column temperature	25°c
7.	Run time	10 minutes

2.4 Method development

2.4.1 Selection and preparation of mobile phase

Various mobile phases containing methanol, water, acetonitrile, and buffer in different ratios were tried with different flow rates. Good symmetrical peak was found with the mobile phase comprising methanol and buffer in the ratio 80:20.

Mobile phase was prepared by taking 400 mL of HPLC grade methanol and 100 mL of TEA buffer and the pH was adjusted to 3.5 with ortho phosphoric acid. The mobile phase was filtered through the 0.45 µm membrane filter and sonicated for 30 min.

2.4.2 Preparation of diluent

80 mL of HPLC grade methanol and 20 mL of HPLC grade water were accurately measured and transferred to 100 mL volumetric flask and then it is sonicated for 10 minutes.

2.4.3 Preparation of dapagliflozin standard stock solution

50 mg of pure drug of dapagliflozin was accurately weighed and transferred into 50 mL volumetric flask and dissolved in 10 mL of the diluent which was previously sonicated and the volume was made up to the mark with the diluent which results in 1000 µg/mL concentration of dapagliflozin stock solution. This solution was degassed in ultrasonicator for 15 minutes.

2.4.4 Preparation of dapagliflozin working standard solution

5 mL of above standard stock solution is taken in a 50 mL volumetric flask and remaining volume is made up to the mark with the solvent which results in 100 µg/mL concentration of dapagliflozin working standard solution.

2.4.5 Preparation of metformin standard stock solution

50 mg of pure drug of metformin was accurately weighed and transferred into 50 mL volumetric flask and dissolved in 10 mL of the diluent (methanol : water in 80 :20 ratio) and the volume was made up to the mark with the solvent which results in 1000 µg/mL concentration of metformin standard stock solution.

2.4.6 Preparation of metformin working standard solution

5 mL of above standard stock solution is taken in a 50 mL volumetric flask and remaining volume is made up to the mark with the diluent which results in 100 µg/mL concentration of metformin working standard solution.

2.4.7 Preparation of standard solution mixture

0.6 mL of metformin stock solution and 0.3 mL of dapagliflozin stock solution were pipetted into a 10 mL volumetric flask and remaining volume was made up to the mark with the diluent which makes 100 µg/mL concentration of mixed standard solution which contains 60 µg/mL of metformin and 30 µg/mL of dapagliflozin.

2.4.8 Preparation of sample solution

Ten tablets were weighed and the average weight of the tablets was noted. The tablets were crushed with a mortar and pestle for 10 minutes. A portion of powder equivalent to the weight of one tablet was accurately weighed and transferred to 100 mL volumetric flask and diluent was added to dissolve and sonicated for 30 minutes and make up the final volume with the diluent. 10 mL of the above solution was filtered through 0.45 µm nylon filter and transferred to 100 mL volumetric flask and diluted to volume with diluent to give test solution containing 30 µg/mL and 60 µg/mL concentrations of dapagliflozin and metformin respectively.

2.4.9 Preparation of calibration curve

From the above working standard solutions, metformin (100 µg/mL) 2, 4, 6, 8, 10 and 12 mL was taken in volumetric flasks and dapagliflozin (100 µg/mL) 1, 2, 3, 4 and 5 mL was transferred into respective 10 mL volumetric flasks and volume was made up to the mark with the solvent to make 10, 20, 30, 40, 50 µg/mL concentrations of dapagliflozin and 20, 40, 60, 80, 100, 120 µg/mL concentrations of metformin.

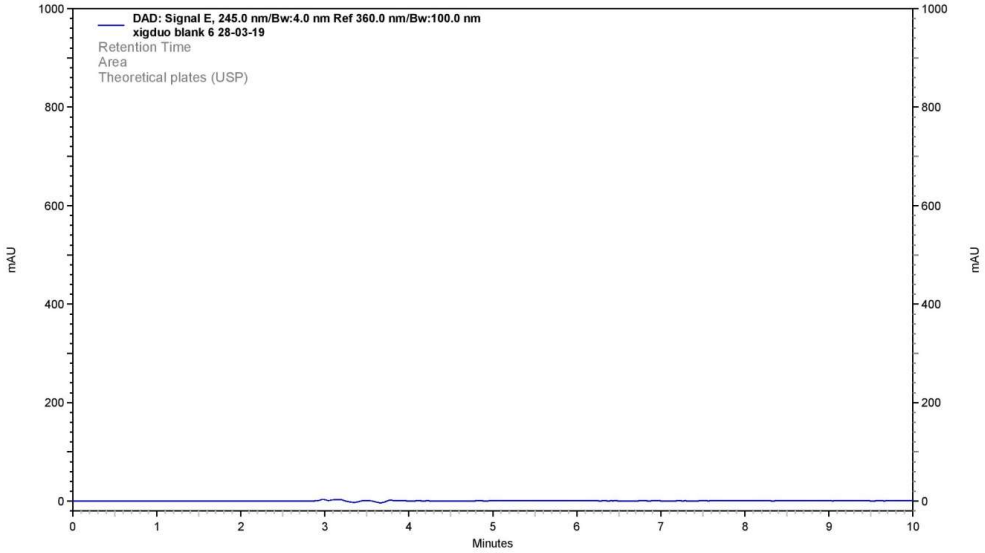

Fig. 3: Chromatogram of blank (Methanol: HPLC grade water)

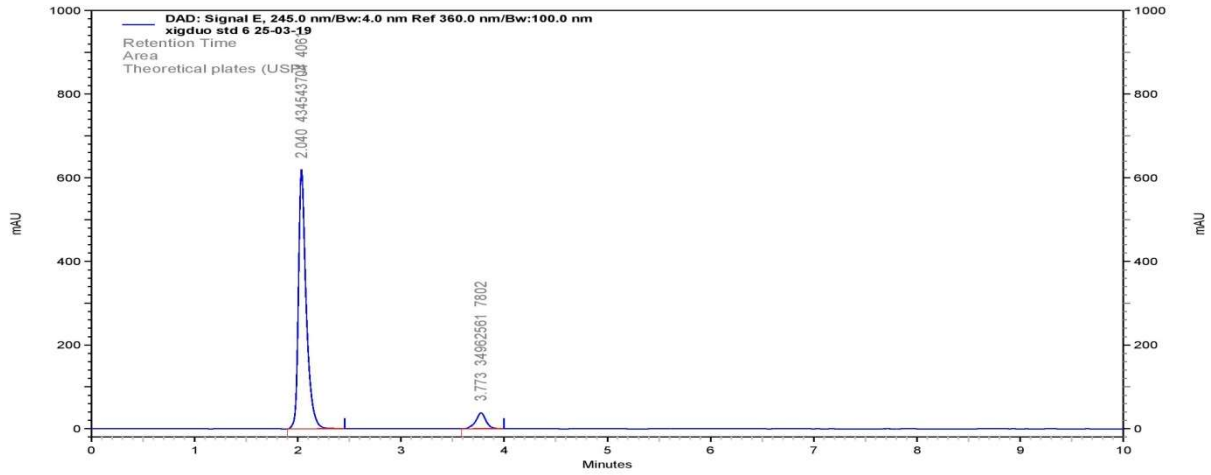

Fig. 4: Chromatogram of standard solution (100 µg/mL) of DAP and MET

2.4.10. Assay of metformin and dapagliflozin formulation

The standard and sample solutions for the formulation were prepared. The samples were analysed with the prepared optimized chromatographic method and the % drug content in the tablets was determined.

$$\%\ Assay = \frac{Peak\ area\ of\ sample}{Peak\ area\ of\ standard} \times \frac{Concentration\ of\ standard}{Concentration\ of\ sample} \times \frac{Label\ claim}{Avg.wt\ of\ tablets} \times \frac{Potency}{100} \times 100$$

2.5 Method validation

The developed method was validated by evaluating linearity, accuracy, precision, robustness, ruggedness, detection limit, quantification limit and stability. Coefficients of variation and relative errors of less than 2 % were considered acceptable, except for the quantification limit, for which these values were established at 2 %, as recommended in the literature

2.5.1 System suitability

System suitability parameters like peak area, retention time, theoretical plates and tailing factor were calculated and were compared with the standard values

2.5.2 Linearity

From the above working standard solutions dilutions were prepared in the range of 10 to 80 µg/mL for dapagliflozin and 20-120 µg/mL for metformin were injected into HPLC. It was shown that the selected drug dapagliflozin had linearity in the range of 10–60 µg/mL and metformin held in the range of 20-120 µg/mL. The calibration plot (peak area ratio versus concentration) was generated by multiple analysis (*n*=6) at all the concentrations of the diluents prepared and was evaluated for a linear relationship using the least square method with Microsoft excel program.

2.5.3 Accuracy

The accuracy of the method was carried out using one set of different standard addition methods at different concentration levels, 50 %, 100 % and 150 %, and then comparing the difference between the spiked value (theoretical value) and actual found value.

2.5.4 Precision

The precision of the method was ascertained from the peak area obtained by actual determination of six replicates of a fixed amount of the drug (60 µg/mL and 40 µg/mL of metformin and dapagliflozin respectively). The precision of the assay was also

determined in terms of intra and inter day variation in the peak areas of a set of drug solutions on three different days. The intra and inter day variation in the peak area of the drug solution was calculated in terms of relative standard deviation (RSD).

2.5.5 Robustness

Robustness of the proposed method was carried out by the slight variation in flow rate, mobile phase ratio and column temperature. The % Recovery and RSD were noted for both the drugs at every level of variation.

2.5.6 Ruggedness

The test solutions were prepared as per test method and injected under variable conditions. Ruggedness of the method was carried out by varying the analysts.

2.5.7 Detection limit and quantification limit

The limit of detection (LOD) and limit of quantification (LOQ) were established based on the calibration curve parameters, according to the following formulas:

LOD= 3.3 SD/slope

LOQ= 10 SD/slope

or detection limit= 3.3 σ/*s*, quantification limit= 10 σ/*s*,

where σ is the standard deviation of *y*-intercept of regression line, and *s* is the slope of the calibration curve [7].

3. RESULTS AND DISCUSSION

The goal of this study was to develop a new RP-HPLC method, several mobile phase compositions were tried for separation and quantification of dapagliflozin and metformin in API and pharmaceutical dosage forms. To develop an effective method for the analysis of the drugs preliminary tests were performed in order to select optimum conditions. Parameters such as detection wavelength, mobile phase composition, column temperature and pH, mobile phase comprising of methanol: buffer in 80: 20 v/v at a flow rate 0.8 mL/min to get a better reproducibility and repeatability. Quantification was achieved with UV detection at 245 nm and the retention time for dapagliflozin and metformin were found to be 2.040 and 3.773 minutes respectively. Typical chromatograms of dapagliflozin and metformin were shown in **Figure No. 3, 4.** The optimized method was validated as per ICH guidelines [8].

3.1 % Assay of the metformin and dapagliflozin formulation

Analysis of metformin and dapagliflozin tablet formulation was performed by the proposed method in the % assay of the formulation was calculated in triplicate. The assay percentage of metformin and dapagliflozin were found to be within limits of 98-102 % in the sample and results are shown in **Table 1**.

Table 1: Assay results of tablet formulation

S. NO	Drug name	Label claim (mg)	Amount found (mg)	Assay (%)*
1.	Metformin	30	29.37	98.0
2.	Dapagliflozin	60	58.98	98.3

* Average assay % of 3 replicate injections

3.2 System suitability

System suitability tests were carried out on freshly prepared standard solutions and the parameters were summarized in **Table 2,3.**

Table 2: System suitability parameters for dapagliflozin

Injection number	Peak area	Theoretical plates	Tailing factor
1	437098313	4059	1.02
2	438766987	4055	1.05
3	437097492	4076	1.04
4	437096629	4148	1.02
5	437090378	4087	1.02
6	437090379	4188	1.04
MEAN	437373363	4102.16	1.03
% RSD	0.156100637	1.24	1.31
Acceptance criteria	NMT 2.0	NLT 2.0	NMT 2.0

Table 3: System suitability parameters for metformin

Injection number	Peak area	Theoretical plates	Retention time
1	36635339	7540	3.073
2	36646856	7590	3.097
3	36683675	7492	3.187
4	36694368	7308	3.049
5	36713261	7547	3.128
6	36665789	7398	3.170
MEAN	36673214.67	7479.167	3.117333
% RSD	0.0803831	1.423021	1.744641
Acceptance criteria	NMT 2.0	NLT 2.0	NMT 2.0

3.3 Linearity

The correlation coefficient for linear curve was obtained between concentration vs. peak area for standard preparations of dapagliflozin and metformin is 0.998 and 0.998 respectively. It shows that the good correlation exist between the drug and response. The results are summarized in the **Table No.4,5.**

Table 4: Linearity data of dapagliflozin

Concentration	Peak area
10	1192745
20	2385491
30	3278237
40	4370983
50	5463728
60	6356474

Table 5: Linearity data of metformin

Concentration	Peak area
20	112117
40	244235
60	336353
80	455879
100	577849
120	682706

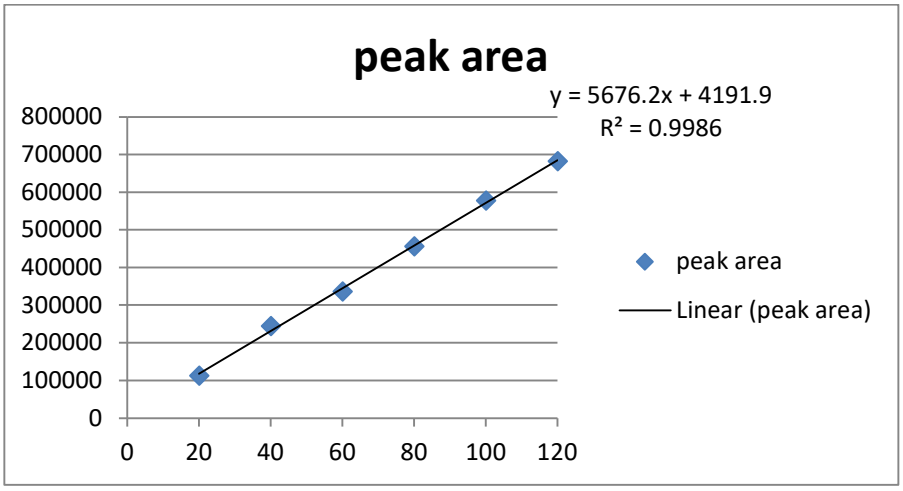

Fig. 5: Linearity plot of dapagliflozin

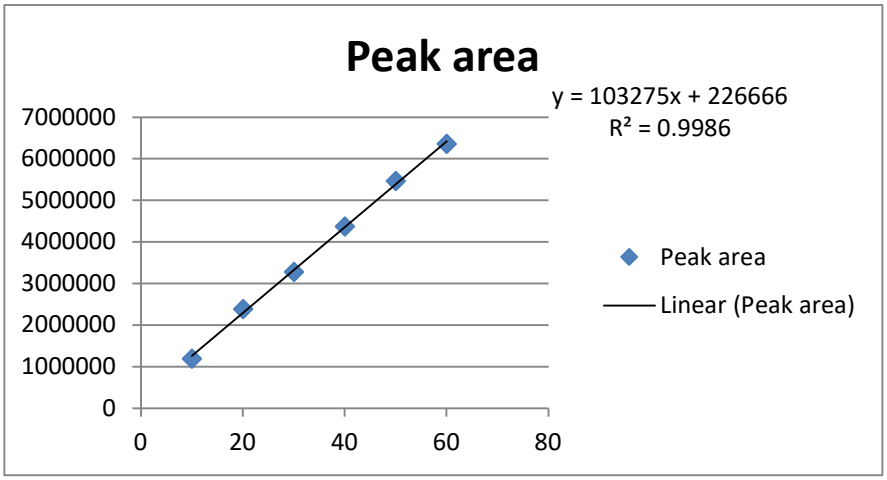

Fig. 6: Linearity plot of dapagliflozin

3.4 Accuracy

The % Recovery for each level obtained for dapagliflozin was found to be within the limits (98-99.9 %). The results obtained for recovery at 50 %, 100 %, 150 % are within the limits. Hence method is accurate. The % Recovery for each level obtained for metformin was found to be within the limits (99-100.2 %) as per the ICH guidelines the results were within the limit. The results are shown in **Table 6, 7.**

Table 6: Accuracy results of metformin

% Conc. at specification level	Standard amount	Amount added	Amount recovered	% Recovery	Mean recovery
50 - level 1	20	9.86	29.04	97.25385131	99.4713173
50- level 2	20	9.45	29.35	99.66044143	
50- level 3	20	9.34	29.78	101.4996592	
100- level 1	20	39.63	59.34	99.51366762	100.2147302
100- level 2	20	39.26	59.92	101.1137361	
100- level 3	20	39.57	59.58	100.016787	
150- level 1	20	69.54	89.12	99.53093589	99.91519655
150- level 2	20	69.25	89.64	100.4369748	
150- level 3	20	69.96	89.76	99.77767897	

Table - 7: Accuracy results of dapagliflozin

% Concentration at specification level	standard amount	Amount added	Amount found	% Recovery	Mean recovery
50- level 2	10	4.76	14.57	97.26301736	
50- level 3	10	4.59	14.84	99.06542056	
100- level 1	10	19.86	29.78	99.73208305	
100- level 2	10	19.74	29.58	99.46200403	98.77325185
100- level 3	10	19.92	29.06	97.12566845	
150- level 1	10	34.83	44.69	99.68770912	
150- level 2	10	34.79	44.83	100.0893056	99.92567159
150- level 3	10	34.24	44.37	100	

3.5. Precision

The % RSD of 6 determinations of dapagliflozin and metformin for system precision of intraday and inter day was found to be within the acceptance criteria of not more than 2.0 %. The results are tabulated in **Table No. 8, 9, 10, 11.**

Table - 8: Intraday precision results of metformin

S. No.	Injection number	Peak area
1	01	36635339
2	02	36673595
3	03	36663450
4	04	36643676
5	05	36732748
6	06	36636790
MEAN		36664266.33
STD DEV		36859.54537
%RSD		0.100532614

Table - 9: Intraday precision results of metformin

S. No.	Injection number	Peak area
1	1	437098325
2	2	436479599
3	3	432552738
4	4	432589076
5	5	431368980
6	6	433578951
MEAN		433944612
STD DEV		2320105.34
% RSD		0.53465472

3.6. Limit of Detection and Limit of Quantification

Limit of detection result for dapagliflozin and metformin was found to be 0.026 and 0.118 respectively and Limit of quantification result for dapagliflozin and metformin was found to be 0.079 and 0.358 respectively and were within the limits. S/N ratio for dapagliflozin and metformin were found to be within the limits. Results are summarized in **Table No. 10, 11.**

Table - 10: Limit of detection values for dapagliflozin and metformin

S. No	Name	Retention time	s/n
1.	Dapagliflozin	2.040	0.026319
2.	Metformin	3.733	0.118146

Table - 11: Limit of quantification values for dapagliflozin and metformin

S. No	Name	Retention time	s/n
1.	Dapagliflozin	2.040	0.079755
2.	Metformin	3.733	0.358019

3.7. Robustness

The analysis was performed in different conditions to fine the variability of test results. The conditions are checked for variation of results. Results are summarized in **Table No. 12, 13.**

Table - 12: Robustness results for Metformin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Change in flow rate				
Actual Flow rate of 0.8 mL/min	36635339	3.733	7640	1.1
Less Flow rate of 0.7 mL/min	36554224	4.252	7381	1.10
Less Flow rate of 0.6 mL/min	35125541	4.858	7536	1.2
Less Flow rate of 0.5 mL/min	35412551	5.122	7225	1.2
More Flow rate of 0.9 mL/min	37521452	3.125	7756	1.1
More Flow rate of 1.0 mL/min	37555522	2.536	7958	1.1
Variation in Mobile phase ratio				
Less organic phase (70:30)	33575263	4.250	7462	1.2
Less organic phase (50:50)	29455425	5.745	6452	1.3
More organic phase (90:10)	37622524	3.225	7762	1.1
Actual ratio of mobile phase(80:20)	36654822	3.773	7642	1.0
Variation in column temperature				
Column temp at 23°c	35254421	4.021	7586	1.2
Column temp at 25°c	36634855	3.774	7652	1.1
Column temp at 27°c	34862122	3.452	7726	1.1
Column temp at 30°c	30254122	3.025	7024	1.3

Table - 13: Robustness results for Dapagliflozin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Change in flow rate				
Actual Flow rate of 0.8 mL/min	437145252	2.046	4058	1.1
Less Flow rate of 0.7 mL/min	423652225	3.124	3856	1.1
Less Flow rate of 0.6 mL/min	425589676	3.659	3468	1.2
Less Flow rate of 0.5 mL/min	402462365	4.125	3158	1.2
More Flow rate of 0.9 mL/min	435895228	1.985	4685	1.1
More Flow rate of 1.0 mL/min	443652645	1.658	5142	1.2
Variation in Mobile phase ratio				
Less organic phase (70:30)	412545221	3.258	3955	1.2
Less organic phase (50:50)	385145220	3.899	3254	1.3
More organic phase (90:10)	448526685	2.012	4256	1.1
Actual ratio of mobile phase (80:20)	437024552	2.042	4098	1.1
Variation in column temperature				
Column temp at 23°c	426542552	2.566	3965	1.2
Column temp at 25°c	437125422	2.040	4021	1.1
Column temp at 27°c	401852111	2.025	4125	1.1
Column temp at 30°c	374554122	1.975	3455	1.3

4. CONCLUSION

The developed HPLC method offers several advantages such as rapidity, usage of simple mobile phase and easy sample preparation steps. From the present study, it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate and reproducible. Results of validation parameters demonstrated that all the results are within limits and the analytical procedure is suitable for its intended purpose. Further, improved sensitivity makes it specific and reliable for its intended use. Hence, this method can be applied for the analysis of pure drug and its pharmaceutical dosage forms.

5. ACKNOWLEDGEMENT

The authors are grateful to the AU college of pharmaceutical sciences, Andhra University, Visakhapatnam for providing the facilities carry out the present research our work. I am also thankful to my fellow classmates.

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