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A QBD driven analytical method development and validation of metformin and dapagliflozin by using reverse phase-high performance liquid chromatography

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***Abstract*---**A simple, sensitive, precise, accurate, and robust, high-performance liquid chromatographic method has been developed to simultaneously estimate Metformin (MET) and Dapagliflozin (DAPA) in pharmaceutical formulation. Design of experiments (DoE) was applied for multivariate optimization of the experimental conditions of the RP-HPLC method. A risk assessment was performed to identify the critical method parameters. The mobile phase composition, flow rate, and column temperature were used to design mathematical models. Box Behnken design (BBD) was used to study the response surface methodology and the effects of these independent factors. The desirability function was used to simultaneously optimize the retention time and resolution of MET and DAPA. The optimized and predicted data from the contour diagram consisted of acetonitrile and ortho phosphoric acid (0.1%) in the ratio of 50:50, respectively, at a flow rate of 1.0 ml/min and column temperature 29°C. Using these optimum conditions, baseline separation of both drugs with good resolution and run time of less than 5 min were achieved. The optimized assay conditions were validated according to ICH guidelines. Hence, the results clearly showed that the Quality by design approach could be successfully applied to optimize the RP-HPLC method for simultaneous estimation of MET and DAPA.

***Keywords*---**design experiments, box behnken design, metformin, dapagliflozin, simultaneous.

Introduction

Metformin hydrochloride

Metformin hydrochloride (Fig.no.1) is 1, 1-Dimethyl biguanide monohydrochloride. It is the first-line medication for the treatment of type 2 diabetes. It is a biguanide ant hyperglycemic agent used for treating noninsulin dependent diabetes mellitus (NIDDM). It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption and increasing insulin mediated glucose uptake. Metformin is the only oral ant hyperglycemic agent that is not associated with weight gain (Afshan Urooj e.t al)

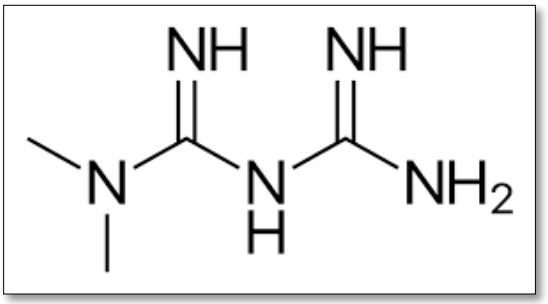

Figure 1 Structure of Metformin

Dapagliflozin

Dapagliflozin (Fig.no.2) is a new oral hypoglycaemic drug approved by theFDA in 2014. Chemically, it is a 2-(4-Chloro-3-(4-ethoxybenzyl)phenyl)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol belonging to the gliflozin class. It is used for the management of type 2 diabetes.Dapagliflozin inhibits sodium-glucose co-transporter-2 (SGLT2) and preventsglucose reabsorption in the kidney. Thelow solubility and stability of dapagliflozin are behind its poor oral bioavailability (Grace a, c et al.,)

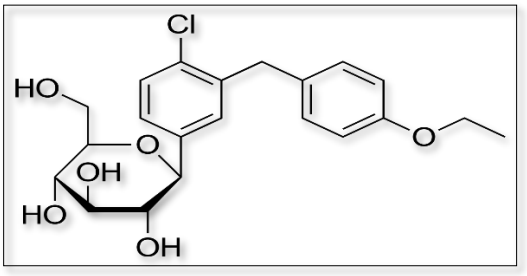

Figure 2 Structure of Dapagliflozin

Materials and Methods

Apparatus: The AZILENT 1290 equipped with a PDA detector , the software installed was OPEN LABS, with 20µl loop, Inertsil ODS 3V(250x4.6mm) 5µm The other instrument included are UV-Visible Spectrophotometer Nicolet evolution

100 Mettler Toledo electronic balance and a sonicator (Citizen, Digital Ultrasonic Cleaner).

Chemicals and reagents

Acetonitrile,Methanol Ortho phosphoric acid – HPLC grade Water for HPLC. All the above chemicals and solvents were supplied by S.D. Fine Chemicals Ltd., India; and Qualigens Fine Chemicals Ltd., Mumbai, India, Merck specialties private limited, Mumbai and Ranbaxy Chemicals Ltd., New Delhi, India.

Methods

Diluent

Buffer: Acetonitrile in the ratio of 50:50 was used as diluent.

Preparation of Buffer: (0.1% OPA)

1 mL of orthophosphoric acid was accurately transferred into a 1000 mL volumetric flask and about 900 mL of milli-Q water added (K. Bhavya's et al). The solution was degassed by sonication and then brought up to the volume (1000 mL) with water (Md. Rageeb Md et al)

Preparation of standard stock solution

About 5 mg of MET and 10 mg of DAPA were accurately weighed and transferred in to a 10 mL clean, dry volumetric flask. The contents of the flask were dissolved in diluent, sonicated for 30 min, and made up to the final 10 mL volume with diluent. From the above stock solutions, 1 mL was pipetted out into another 10 mL volumetric flask and then made up to the 10 mL volume with diluent.

Sample preparation

10 tablets were weighed and crushed. From that, a powder equivalent to 5 mg and 10 mg of MET and DAPA was weighed accurately and transferred into a10 mL clean dry volumetric flask. The contents of the flask were dissolved in diluent, sonicated for 30 min, and made up to the final volume with diluent and labeled as Sample stock solution. Sample stock solution was filtered by PVDF 0.45um filters. 1 mL of filtered sample stock solution was transferred to 10 mL volumetric flask and made up to the volume (10 mL) with diluent.

Chromatographic procedure

Chromatographic separations were carried out on a Discovery C18 column (250 mm, 4.6 mm, and 5 μm). A mixture of acetonitrile and 0.1% orthophosphoric acid (50:50) was used as the mobile phase. Wavelength of 210 nm was used for detection, at which both drugs gave good response(Fig 3).

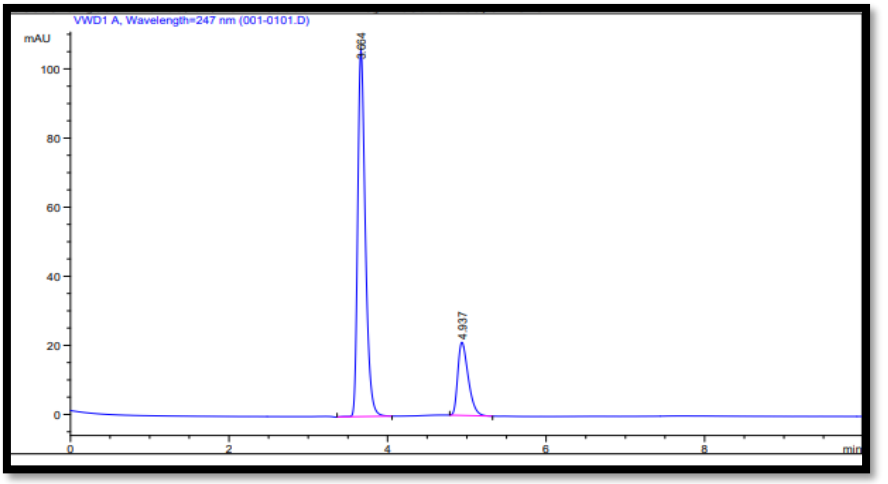

Figure 3 Chromatogram of optimized peak

Method validation

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) (2005) Q2R (1) guidelines for system suitability, linearity, limit of detection, limit of quantitation, precision, accuracy, specificity, and robustness.

System suitability test

According to United States Pharmacopoeia (USP), system suitability tests are integral part of liquid chromatographic methods. System suitability parameters, like number of theoretical plates, resolution, and tailing factor were evaluated by injecting six replicates of standard solutions containing 50 µg/mL of MET and 100 µg/mL of DAPA before the sample analysis. In all cases, the percent relative standard deviation should be < 2.0%. The acceptance criteria for standards in system suitability were set in each chromatogram

Linearity

Linearity of the developed method was established at six levels over the range of 20-100 µg/mL for MET and 50-150 µg/mL for DAPA. Each linearity solution of respective sample concentrations was injected in triplicate. The calibration curve was constructed by plotting the peak area against the concentration, using linear regression analysis.The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of metformin and Dapagliflozin is 0.995 and 0.999. The relationship between the concentration and area of Metformin and Dapagliflozin is linear in the range examined since all concentration and area of Metformin and Dapagliflozin is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

Table 1 Linearity of Metformin

S.No.	Conc. (µg/ml)	Area
1	20	3236.788
2	40	4409.861
3	60	5560.106
4	80	6760.326
5	100	7803.508

Table 2 linearity of Dapagliflozin

S.No.	Conc. (µg/ml)	Area
1	50	3689.12
2	80	5308.85
3	100	6374.88
4	120	7505.29
5	150	8963.14

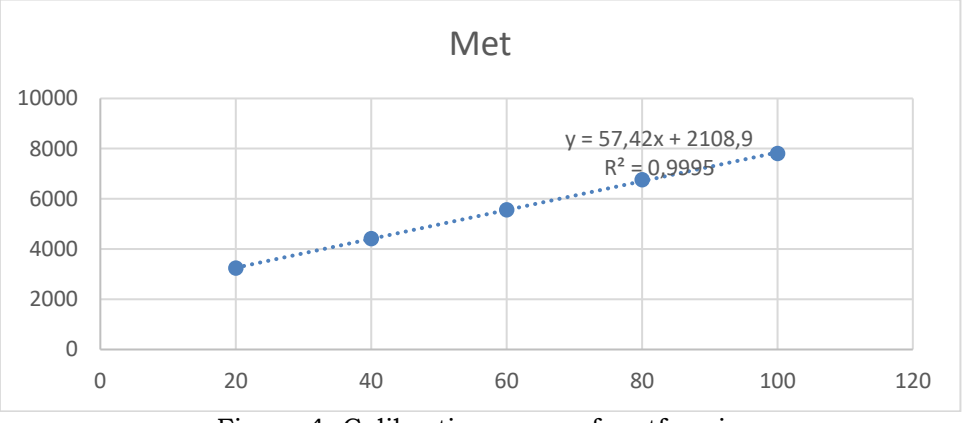

Figure 4 Calibration curve of metformin

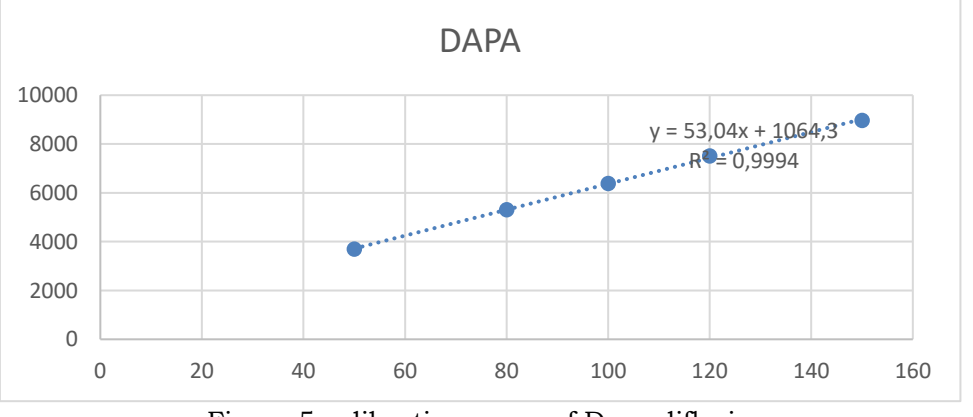

Figure 5 calibration curve of Dapagliflozin

Accuracy and precision

Accuracy was carried out by adding a known amount of standard to the tablet solution for each drug at 50, 100, and 150 % levels in triplicate, and samples were analyzed by the optimized method. Percentage recovery was then calculated for both drugs. The mean recovery of the target concentrations was set to 100 ± 2% for acceptance Precision of the optimized method was determined by studying the intermediate precision and repeatability. Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. Intermediate precision is also known as inter-assay precision. Repeatability expresses the precision under the same operating conditions over a short interval of time Six homogenous samples of MET and DAPA were assayed to assess the method precision

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of MET and DAPA were evaluated using the standard deviation method. LOD was defined as 3.3 σ/S and LOQ as 10 σ/S based on standard deviation of the response (σ) and slope of the calibration curve (S).

Robustness

The robustness of the method refers to its ability to remain unaffected by small and deliberate variations in method parameters. The robustness of the optimized method was investigated by injecting the system suitability solution with minute deliberate changes in the chromatographic parameters, flow rate (0.97-0.99 mL/min), proportion of solvent in mobile phase (45:55) and temperature of the column (30.04-32.24 °C). It was measured on the basis of percent relative standard deviation.

Method Validation

Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-sample solution

Table 3 Recovery results of metformin

Recovery level	Accuracy Metformin			Average% Recovery
	Area	Average area	%Recovery	
80%	5822.5	5826.06	100.03	
	5825.2			
	5830.5			
100%	6392.12	6392.12	101.30	100.17

	6394.18			
	6392.21			
120%	7119.21	7120	99.2	
	7116.32			
	7125.25			

Table 4 Recovery results of Dapagliflozin

Recovery level	Accuracy of Dapagliflozin			Average % Recovery
	Area	Average area	%Recovery	
80%	6735.0	6736.0	101.3	101.0
	6738.2			
	6735.8			
100%	7421.2	7422.1	100.1	
	7423.3			
	7422.1			
120%	8118.2		100.00	
	8119.5			
	8122.5			

Precision

Precision is measured by injecting a series of standards or analyzing series of samples from multiple samplings from a homogeneous lot.

Table 5 Precision of metformin

S.No.	Area
1	3236
2	3234
3	3235
4	3236
5	3233
Avg	3234
Stdev	1.16
%RSD	0.03

Table 6 Precision of Dapagliflozin

S.No.	Area
1	3689
2	3684
3	3685
4	3692
5	3686
avg	3686
stdev	3.2
%RSD	0.08

Robustness

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision

Table 7 results of robustness

	Metformin		Dapagliflozin	
Parameter	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow Rate				
0.8 ml/min	4.353	1.37	4.643	1.41
1.2 ml/min	3.823	1.29	4.683	1.38
Wavelength				
220nm	3.833	1.42	3.610	1.41
235nm	3.827	1.41	3.580	1.43

Assay:
The amount of Metformin and Dapagliflozin present in the taken dosage form was found to be 100.04% and 100.36% respectively.

QBD-OPTIMIZATION

For the RP-HPLC method, a multivariate approach DoE with BBDwas applied to study the simultaneous variations of the factors on considered responses, such as retention time of MET (Y1), retention time of DAPA (Y2), and resolution (Y3) to test method robustness. Based on the effects of three factors on responses and evaluation of these results, it was feasible to elaborate mathematical models that had been endeavored to find out the relationship between the factors and the responses of interest studied. We observed that the best fitted model for BBD was the response surface quadratic model. The model was also validated by ANOVA using Design Expert software. The predicted R- squared values of retention time of MET (Y1) and DAPA (Y2) were in reasonable agreement with adjusted R- squared values i.e., the difference is less than 0.2, A negative predicted R- squared value for resolution (Y3) implies that the overall mean may be a better predictor of the response than the current model. Adequate precision, measures the signal to noise ratio. A ratio of greater than 4 is desirable and the obtained responses for the Y1, Y2, and Y3 were 96.445, 29.953, and 6.118, respectively, which indicates an adequate precision. This quadratic model can be used to navigate the design space. Model F-value of responses for retention time of MET (Y1), retention time of DAPA (Y2), and resolution (Y3) were 666.4, 70.44, and 4.45, which implies the model is significant. There is only a 0.01% chance, in the case of Y1 and Y2, while a 1.44% chance for resolution (Y3) than an F-value, indicating that this could occur due to noise. Hence, the values of significant responses showed p value < 0.05, suggesting that the model terms are significant. The low standard deviation

and high adjusted R-square value indicates a good relationship between experimental data and those of fitted models. The equations in terms of coded factors can be used to make predictions about the response for given levels of each factor. This equation is useful for identifying the relative impact of the factors by comparing the factor coefficient.
Final equations for Y1, Y2, and Y3 are: MET (Y1) = +2.77 - 0.063X1 - 0.28X2 - 2.95X3 + 9.87X1X2 + 6.87X1X3 + 2.87X2X3 + 9.15X1 2 + 0.03X2 2 + 6.50X3 2 ; DAPA: (Y2) = +3.41 - 0.08X1 - 0.32X2 - 0.08X3 - 0.03X1X2+ 0.01X1X3 - 0.04X2X3 + 0.01X1 2 + 0.044X1 2 + 0.06X3 2 ; Resolution: (Y3) = +3.60 + 0.02X1 + 0.01X2 - 0.03X3 - 0.15X1X2 + 0.10X1X3 - 0.17X2X3 + 0.06X1 2 + 0.06X2 2 + 0.34X3 2 . As per the values of coefficient from the above equations and their signs, it is clear that factors, such as mobile phase composition (X1), flow rate (X2), and column temperature (X3), had a negative effect on retention time of MET and DAPA, Y1 and Y2. The column temperature (X3) had a negative effect on resolution (Y3), whereas mobile phase (X1) and flow rate (X2) had positive effects. Interactions of X1 and X2 had a positive effect on Y1 and Y2 and a negative effect on Y3; X2 and X3 had a positive effect on Y1 and a negative effect on Y2 and Y3; X1 and X3 had a positive effect on Y1 and Y2 and a negative effect on Y3. The squares of factors, X1 2, X2 2, and X3 2, had positive effects on all chromatographic responses. Response surface and contour plots were analyzed to visualize the effect of the factors and their interactions on the responses The contour plots showed curvature, displaying a nonlinear effect of factors on responses.) contour plots displaying the effect of mobile phase ratio (X1) and flow rate (X2) on retention time of MET (Y1) and DAPA (Y2). A curvilinear increasing trend was observed for the mobile phase ratio (X1) and flow rate (X2), which showed higher resolution time of MET (Y1), as well as DAPA (Y2) at lower levels. Therefore, lower levels of X1 and X2 were recommended to achieve high retention time of MET (Y1) and DAPA (Y2). Therefore, optimized levels of X1 and X2 were recommended to achieve resolution. A composite desirability was applied to obtain an optimum set of conditions based on the specified goals and boundaries for each response. The desirability function "R", equal to unity, demonstrated the achievement of desired goals in the constraints set and the whole experimental area was explored for the compositions where in constraints set were met to the maximum i.e., unity, as shown in Figure 6. The optimum values of chromatographic conditions of RP-HPLC were selected as mobile phase (X1) buffer and acetonitrile 50:50, flow rate (1.0 mL/min), and column temperature 29 °C which resulted in retention time of MET (Y1) 2.79±0.0162, retention time of DAPA (Y2) 3.45±0.013, and resolution (Y1) 3.7±0.002 min, respectively,

Table 8 – Factors

FACTORS	CODE	LOW	HIGH
Flow rate	X1	0.9	1.2
Mobile phase	X2	49	50
Column Temperature	X3	29	30

Table 9 Response

Run	Factor-1 Flow rate ml/min*(X1)	Factor-2 Mobile phase composition(X2)	Factor-3 Temperature(X3)	Response -1 Retention time of Met (Y1)	Response-2 Retention time of Dapa(Y2)
1	0.1	50	29	3.12	4.12
2	0.12	50	30	3.10	4.03
3	0.1	55	29.5	3.02	4.15
4	0.11	50	29.5	3.08	4.15
5	0.11	45	29	3.06	4.35
6	0.11	50	29.5	3.56	4.14
7	0.11	50	29.5	3.25	4.27
8	0.11	50	29.5	3.28	4.48
9	0.11	45	30	3.02	4.38
10	0.12	45	29.5	3.12	4.94
11	0.12	55	29.5	3..15	4.03
12	0.11	55	30	3.18	4.32
13	0.1	45	29.5	3.89	4.01
14	0.11	55	29	3.58	4.10
15	0.11	50	26.5	3.15	4.12
16	0.1	50	30	3.16	4.12
17	0.1	50	29	3.04	4.14

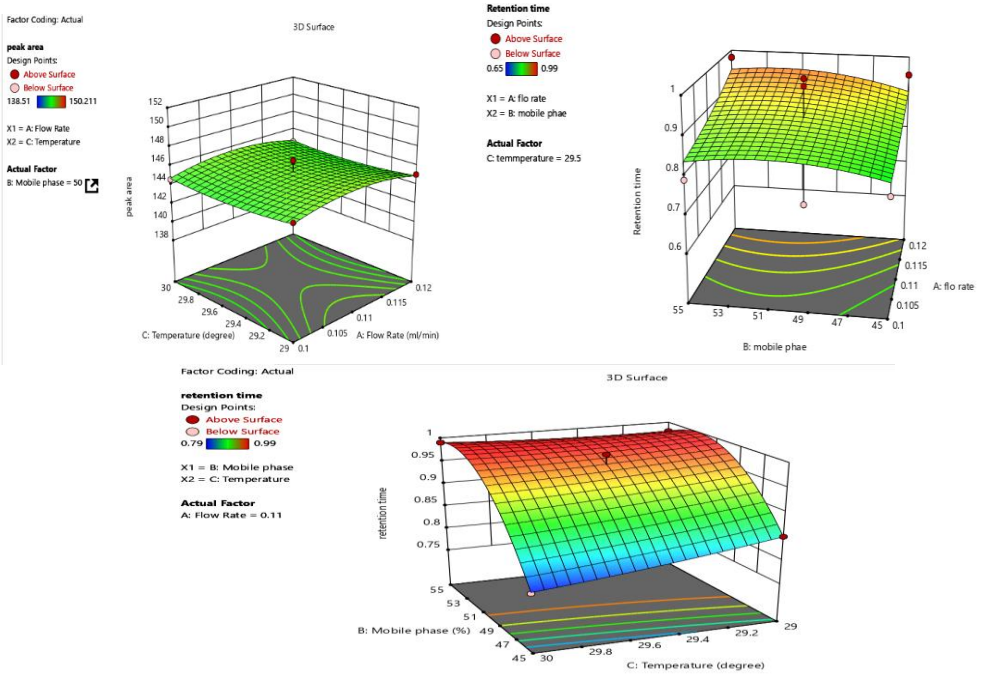

Figure 6 3D-response surface plots depicting the influence of mobile phase ratio, flow rate and temperature on the retention time as the response variable
Robustness testing using experimental design

Conclusion

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation Metformin and Dapagliflozin was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories studies in near future. The experimental design describes the scouting of key components, including mobile phase composition, flow rate, and column temperature. The modeling software facilitated better understanding of the factors influencing optimization of the method and separation of MET and DAPA. BBD was applied to optimize the resolution as response between MET and DAPA in a relatively short time (5 min). In the optimized model, the acetonitrile and orthophosphoric acid (0.1%) in the ratio of 50:50 indicates the suitability for estimation of MET and DAPA. The flow rate of the mobile phase was optimized at 1.0 ml/min and column temperature at 29 °C respectively

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