APPLICATION OF DOE IN DEVELOPING A VALIDATED RP-HPLC METHOD TO EVALUATE DAPAGLIFLOZIN AND METFORMIN IN COMBINED TABLET DOSAGE FORM

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Abstract
In the current investigation, a statistical experimental design was used to create an isocratic RP-HPLC method for the simultaneous quantification of dapagliflozin and metformin in bulk and tablet dosage form. Acetonitrile concentration, buffer pH, and flow rate were used as three independent design variables. To thoroughly analyse the effects of these independent elements and analyse the response surface technique, Central Composite Design (CCD) was utilised. Derringer's desirability function was used to simultaneously optimise these three replies. ACN: Menthol (40:40:20) %V/V (pH-2.6) as the mobile phase and a flow rate of 1.0ml/min were the ideal test conditions. In order to verify the optimised procedure's specificity, linearity, accuracy, and precision, it was verified in accordance with ICH requirements.

Keywords: Chromatography, Central composite design,Response surface methodology, Optimization, Method validation

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INTRODUCTION

Chromatography is a non-degradative method that separates a multicomponent mixture into fractions of minor, minor, or major components. Different variations can be applied to solids, liquids and gases. Although chromatography can be applied quantitatively, it is primarily a separation tool. High-Performance Liquid Chromatography (HPLC) is an analytical separation technique and is considered the gold standard used by almost all analytical laboratories in the pharmaceutical industry during the life cycle of a pharmaceutical product. Because HPLC uses a wide variety of chromatographic factors, namely the type and concentration of organic modifier, pH, buffer molarity, temperature, flow rate, etc., and the simultaneous optimization of resolution and analysis time, the optimization of experimental conditions is difficult process.

As a result, method development has typically been accomplished by changing one factor at a time (OFAT) or by using a more systematic technique, such as software programmes and design-of-experiments, as an effective and quick instrument for method development. The developed method is optimised in the current study using the DOE approach. This entails selecting the method with the highest accuracy and specificity for routine QC analysis, followed by an assessment of the factors affecting separation, such as resolution, retention time, and tailing factor, as well as the development of the method using a statistical model with a relationship between the factors and the response. In order to determine the ideal flow rate, mobile phase concentration, and pH, the chromatographic parameters were chosen based on knowledge from the literature review and optimised using central composite design.. For three independent variables, a partial factorial design with five replicates of center points and five axial points is combined. From the results, the qualities of the fitted second-order polynomial models were calculated using the coefficient of determination. Then by applying derringers desirability function the flexible optimized chromatographic conditions were selected for the determination of drugs in a variety of samples.

Dapagliflozin and metformin combination is used together with proper diet and exercise to treat type 2 diabetes. It is also used to lower the risk of hospitalization for heart failure in patients with type 2 diabetes and cardiovascular (heart or blood vessel) disease or multiple cardiovascular risk factors. This medicine is also used to lower the risk of cardiovascular death and hospitalization in patients with heart failure with reduced ejection fraction (the heart is weak and cannot pump enough blood to the rest of the body. Metformin is a component of a variety of combination products with other anti-diabetic agents. It is indicated, along with diet and exercise, to improve glycemic control in adult patients with type 2 diabetes mellitus in combination with DPP-4 inhibitors like sitagliptin, linagliptin, alogliptin, or saxagliptin), in combination with SGLT2 inhibitors (canagliflozin, empagliflozin, ertugliflozin, or dapagliflozin), or in combination of these two drugs Dapagliflozin and Metformin is avalilable as Xidguo XR 10/500mg which is mainly used in the treatment of diabetics mellitus.

The literature review reveals that only a few methods are available for the simultaneous estimation of these three drugs by spectroscopy using chemometrics. No method has been reported for the simultaneous estimation of the Dapagliflozin and metformin using chemometrics. Hence an attempt was made to develop, optimize and validate an accurate and sensitive HPLC method for the simultaneous determination of the above drugs in tablet dosage form using experimental design.

MATERIALS AND METHODS

Chemicals and reagents

Pure standards of Dapagliflozin (DAPA) and Metformin (MET) were gifted from Nebulae Hi-Tech Chennai. The tablet formulation Xigduo XR containing Dapagliflozin DAPA (10mg) and Metformin MET (500mg) was purchased from a local pharmacy. Methanol (AR grade), Methanol (HPLC grade), Acetonitrile (HPLC grade), Water (HPLC grade), Orthophosphoric acid (AR grade), and triethylamine (AR grade) were purchased from Loba chemical India Limited, Mumbai.

Instrumentation

Chromatographic measurements were made on Shimadzu HPLC having detector with deuterium lamp source in the range 280nm with double reciprocating plunger pump with constant flow and pressure delivery. The mobile phase was degassed by using Ultrasonicator (3.5L100) Ikon Industries Ultra sonic bath. The UV spectrum was recorded using a UV-Visible spectrophotometer (Model Shimadzu, Japan (Model UV 1800) - softwareUV probe 2.32 version.

Software
Experimental design, data analysis, and desirability function calculations were performed by using Design -Expert trial version 12.0 (State- Ease Inc., Minneapolis). The rest of the calculations for the analysis were performed by the use of Microsoft excel 2007 software (Microsoft USA).

Mobile phase selection
The main requirement of the mobile phase is that it has to dissolve the analytes up to the concentration suitable for detection. The mobile phase absorbance should usually be less than 0.5 at the wavelength used for detection. When the absorbance of the mobile phase exceeds a value of about 1.0 the detector may become unusable. Hence the mobile phase suitable for samples is selected by performing trials with different ratios of the mobile phase.

Preparation of Mobile phase
The mobile phase was prepared by mixing 50.0 ml of acetonitrile with 50.0 ml of 1% triethylamine (pH adjusted to 2.6 with orthophosphoric acid). This mobile phase was filtered through a 0.42 µ membrane filter and then it was ultra-sonicated for 15 minutes.

Preparation of standard stock solution
About 10 mg of the reference standard of DAPA and 500mg of the reference standard of MET were accurately weighed separately and transferred into 50 ml volumetric flasks. The drugs were dissolved in 50 ml of Methanol with shaking and then the volume was made up to the mark with the methanol. Finally, the concentration of the solution was to get 200 µg/ml for DAPA and 1000µg/ml for MET.

Preparation of Sample solution
Marketed tablet formulation Xigduo XR tablet contain 10mg of DAPA and 500mg of MET. Ten tablets were weighed accurately; the average mass per tablet was determined and finely powdered. The powder equivalent to 500mg of MET was accurately weighed and transferred into a 50 ml volumetric flask containing 25 ml of mobile phase and then ultrasonicated for 20 min. Finally, the volume was made up to themark with methanol. The solution was filtered through Whatman filter paper. Insouble excipients were separated out. The filtrate was collected after rejecting the first portion of the filtrate. 0.5ml of the clear solution was further diluted and made up to 10ml with mobile phase to obtain 5µg/ml for DAPA and 250µg/ml of MET). 20 µl of each solution was injected and the chromatogram was recorded. The analysis was repeated six times. The content of the drug was calculated from the peak area recorded.

Selection of wavelength
For many samples, good analytical results will be obtained only by careful selection of the wavelength used for detection. The sensitivity of HPLC depends upon the proper selection of the wavelength of detection. To determine the proper wavelength of Dapagliflozin (DAPA) and Metformin (MET) in the mobile phase, spectra were scanned on UV-Visible spectrometer in the range of 200-400 against diluent as blank. The Isobestic point of wavelength 215nm was selected for the analysis.

Method Validation
The RP-HPLC method was validated in terms of parameters like accuracy, linearity, precision, range, detection limit, quantification limit, ruggedness, robustness and systemsuitability, etc. For all the parameters percentage relative standard deviation values were calculated.

RESULTS AND DISCUSSION
To understand the sensitivity of the chromatographic factors on the separation of analytes and for simultaneous optimization of resolution and analysis time, Chemometric protocols of Response surface design and Derringer's desirability function were successfully employed. The central composite design can be applied to optimize the separation and to assist the development of a better understanding of the interaction of several chromatographic factorson separation quality. The selection of factors for optimization was based on preliminary experiments and prior knowledge from the literature. Therefore, the key factors selected for the optimization process were Acetonitrile concentration (*A*), Buffer pH (*B*), and Flow rate (*C*). Table 1 shows the levels of each factor studied for finding out the optimum values and responses. The ranges of each factor used were MeOH concentration (70-30%), Phosphate buffer pH (2.5), and Flow rate (1.0mL/min). As response variables, the capacity factor of Hydrochlorothiazide (*k*1), the resolution between two pairs amlodipine and telmisartan (Rs1, 2), and the retention times of Dapagliflozin (Rt2) were chosen. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that mayintroduce a bias on the measurements.

Central composite design with quadratic equation was represented as

Table 6.3 Experimental design- CCD – Analysis of Dapagliflozin &Metformin

Run	Space type	FACTORS			RESPONSE		
		A MeOH conc (%)	B Buffer pH	C Flow rate (ml/min)	1 Capacity factor (K_1)	2 Retention time (Rt_2)	3 Resolution ($Rs_{1,2}$)
1.	Axial	41.591	2.5	1.00	1.12	3.09	7.14
2.	Center	50	2.5	1.00	1.11	4.42	5.934
3.	Factorial	45	3	1.2	1.16	7.65	9.772
4.	Factorial	45	3	0.8	1.39	6	8.73
5.	Factorial	55	2	1.2	1.15	4.06	8.289
6.	Factorial	45	2	0.8	1.15	5.41	8.237
7.	Center	50	2.5	1	1.11	4.42	5.934
8.	Axial	58.409	2.5	1	1.11	3.84	8.034
9.	Axial	50	2.5	1.33636	1.15	4.31	7.448
10.	Factorial	45	2	1.2	1.12	6.28	8.541
11.	Factorial	55	2	0.8	1.19	5	8.743
12.	Center	50	2.5	1	1.11	1.42	5.934
13.	Axial	50	2.5	0.66364	1.16	.72	8.694
14.	Axial	50	1.6591	1	1.14	4.64	8.662
15.	Axial	50	3.3409	1	1.15	7.73	8.792
16.	Factorial	55	3	1.2	1.14	6.8	7.921
17.	Center	50	2.5	1	1.11	4.42	5.934
18.	Center	50	2.5	1	1.11	4.42	5.934
19.	Center	50	2.5	1	1.11	4.42	5.934
20.	Factorial	55	3	0.8	1.2	3.53	6.837

$Y = \beta0 + \beta1\ X1 + \beta2\ X2 + \beta3\ X3 + \beta12\ X1\ X2 + \beta13\ X1\ X3 + \beta23\ X2\ X3 + \beta11\ X12 + \beta22\ X22 + \beta33\ X32$

$X3^2$where Y is the response to be modeled, β is the regression coefficients and X1, X2and X3 represent factors A, B and C respectively. Statistical parameters obtained from ANOVA for the reduced models were given in table 2. The insignificant terms ($p>0.05$) were eliminated from the model through a backward elimination process to obtain a simple and realistic model. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected.

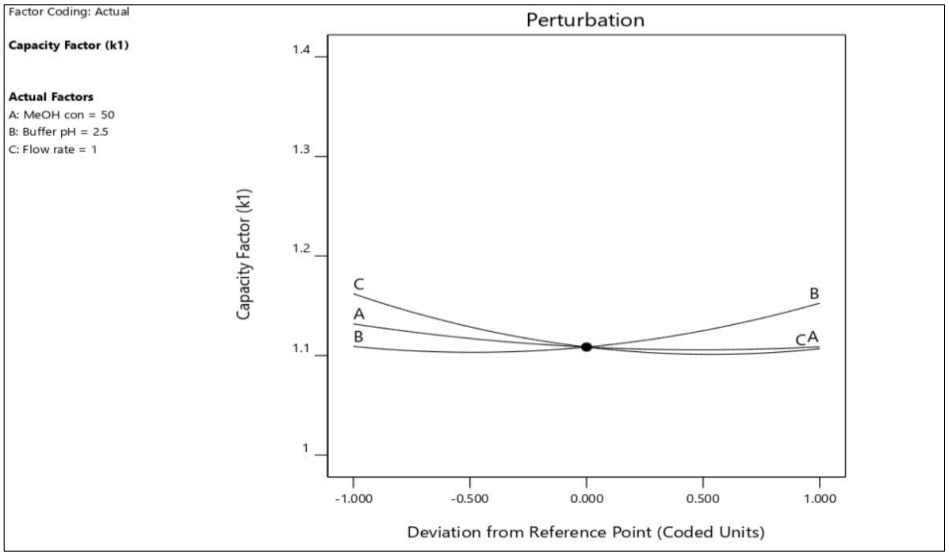
Table 2 Reduced Response Surface Models and Statistical Parameters obtained from ANOVA

Responses	Regression model	Adjuste R^2	Model p value	% C.V	Adequate Precision
K_1	+1.11 -0.0115A + 0.0217B - 0.0276C -0.0350AB + 0.0200AC - 0.0275BC + $0.0118A^2$ + $0.0224B^2$ + $0.0259C^2$	0.4638	0.0001	4.00	6.4100
Rt_1	+4.41- 0.3433A+0.6170 B - 0.0648C - 0.0862AB -0.0237AC + 0.6238BC -$0.26998A^2$ + $0.6918B^2$ + $0.6316C^2$	0.2811	0.0001	23.45	5.0692
$RS_{1,2}$	+5.93-0.1455A-0.0193B-0.0088C -0.4997AB -0.0895AC + 0.2845BC +$0.6117A^2$ +$1.01B^2$ + $0.7828C^2$	0.8081	0.0001	7.33	8.6513

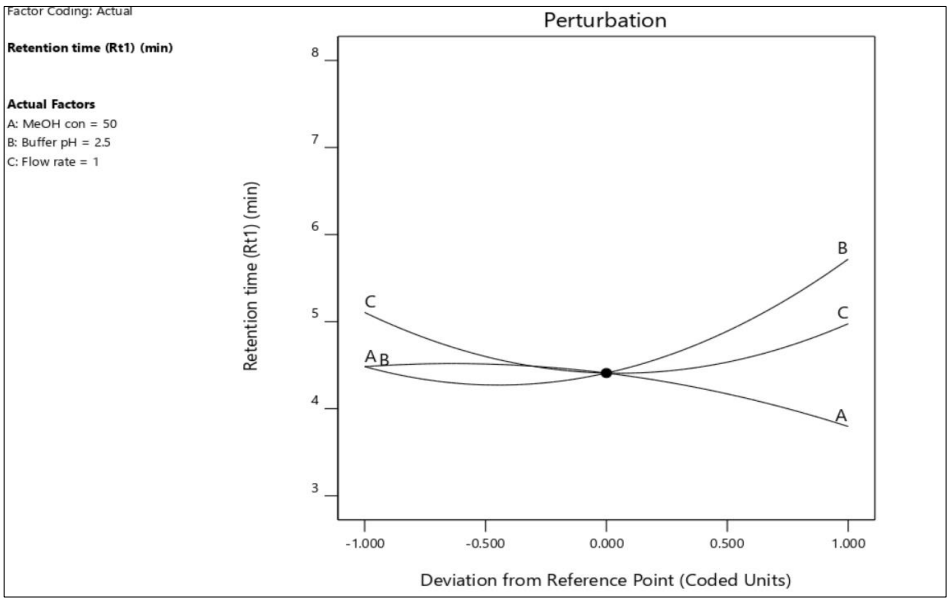
The adjusted R^2values were well within the acceptable limits of $R^2 \geq 0.80$ which revealed that the experimental data showed a good fit with second-order polynomial equations. For all the reduced models, the *p*-value of < 0.05 was obtained, implying these models were significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio.A ratio greater than 4 is desirable. The ratio was found to be in the range of 4.00 – 23.45 whichindicated an adequate signal and therefore the model was significant for the separation process. The

coefficient of variation (C.V) is a measure of reproducibility of the model and asa general rule, a model can be considered reasonably reproducible if it is less than 10%.

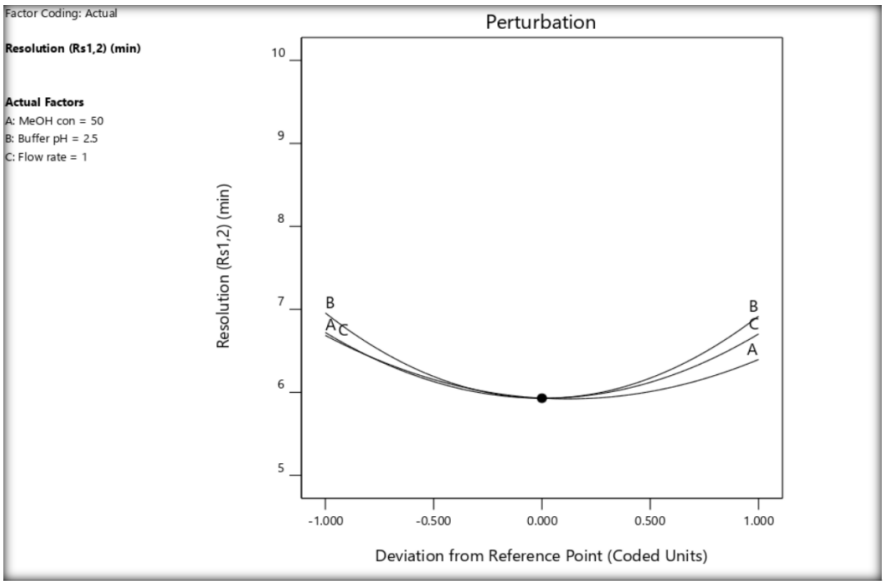
In table 2 the interaction terms with the largest term coefficient among the fitted model were BC (+ 0.25) of $Rs_{2,3}$ model. The positive interaction between B and C was statistically significant (< 0.0001) for $Rs_{1,2}$. The existence of such interactions emphasizes the necessityto carry out active multifactor experiments for the optimization of chromatographic separation. To gain a better understanding of the results the predicted models were presented in the form of perturbation plot figure 1 and 3D response surface plot figure 2. Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. Consequently, factors A and Cwere selected for the response plots of k_1, $Rs_{1,2}$, and Rt_2 with factor B held constant usually at a central value of buffer pH 2.6. All these three-dimensional plots were beneficial to gain an overall understanding of the influence of phosphate buffer pH and flow rate on analysis time ($Rs_{1,2}$). Perturbation plots provide silhouette views of the response surface plots, where it shows how the response changes as each factor move from a chosen reference point, with all other factors, held constant at the reference value.



(A)Capacity factor

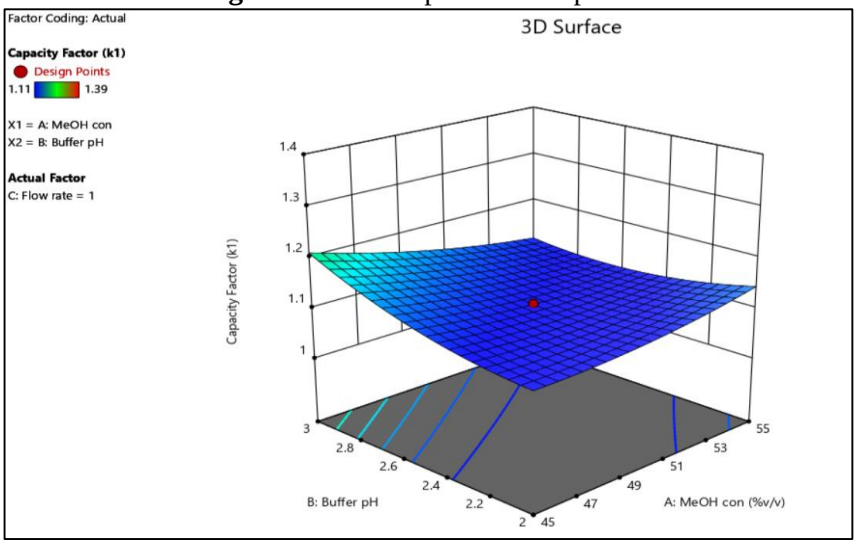


(B) Resolution

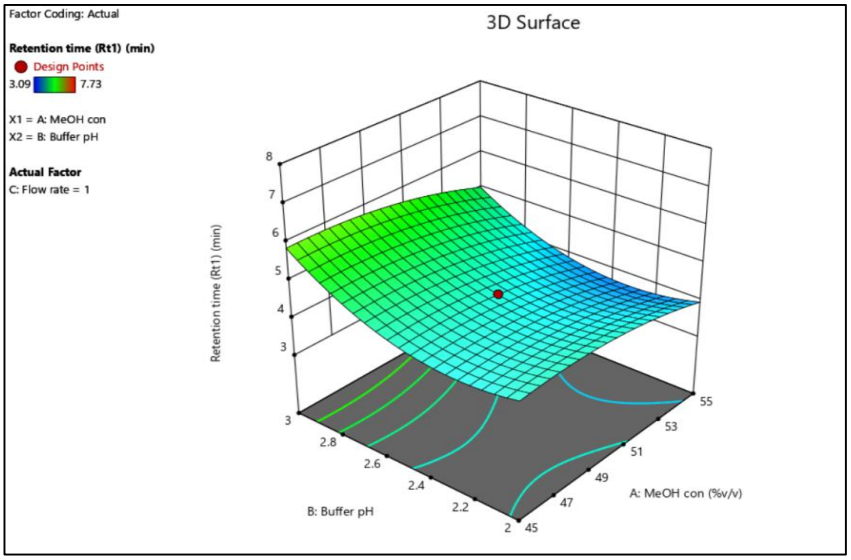


(C) Retention time

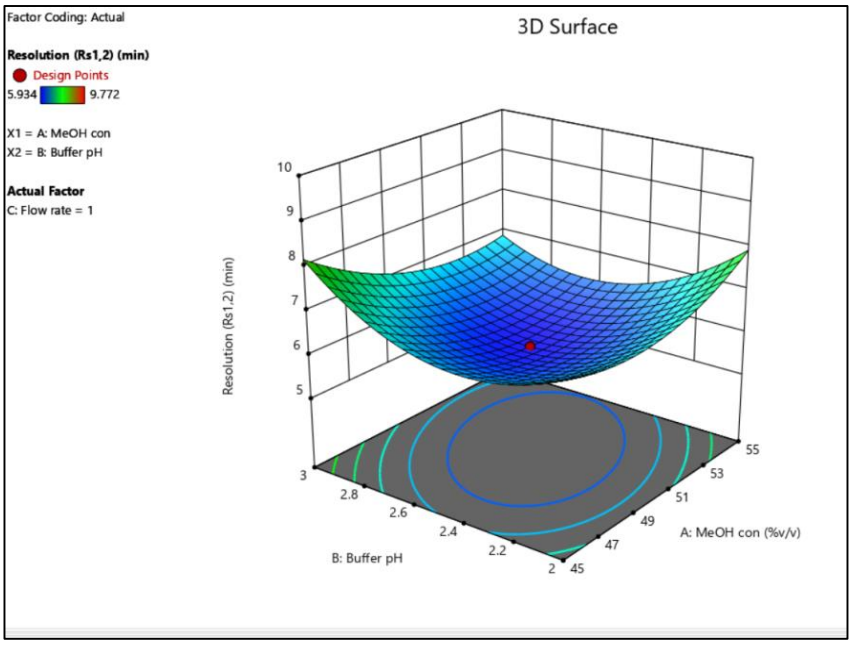
Fig 1 Perturbation plots for Responses



(a) Capacity factor



(b) Resolution



(c)Retention time

Fig 2 Response surface plots for Responses

The steepest slope or curvature indicates the sensitiveness of the response to a specific factor. Figure 2b showed that 1% triethylamine buffer pH (factor B) had the most important effecton resolution between Amlodipine and Telmisartan $Rs_{2,3}$ followed by factor C and then factor-A. The rest of the factors (MeOH concentration and flow rate) had a significant effect on tR_3 and k_1. When k_1 and tR_3 values were increased, the level of MeOH concentration (factor A) increased, and when k_1 and tR_3 values decreased, the level of flow rate (factor C) increased. Analysis of the perturbation plot and response surface plot of optimization models revealed that factors B and C had a significant effect on the separation of analytes, whereas the factor A, MeOH concentration was of little significance. The criteria for the optimization of each response were shown in table 3.

Derringer's desirability function was employed for the global optimization of three responses and to select different optimal conditions for the analysis of formulation in the present study. The identified criteria for the optimization were resolution between the peaks, capacity factor, and elution time.

Derringer's desirability function, D, is defined as the geometric mean, weighted or otherwise of the individual desirability functions. The expression that defines Derringer's desirability function is:

$$D = [d_1^{p2} \times d^{p2} \times d^{p2} \times \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\times d_n^{pn}]^{1/n}$$

where pi is the weight of the response, n is the number of responses and di is the individual desirability function of each response. Desirability function (D) can take values from 0 to 1. Weights can range from 0.1 to 10. Weights lower than 1 give less importance to the goal, whereas weights greater than 1 give more importance to the goal. The criteria for theoptimization of each response were shown in table 3.

Table 3 Criteria for the Optimization of the Individual Responses

Factors/Responses	Lower limit	Upper limit	Criteria/Goal
A: MeOH Conc	45	55	is in range
B:Buffer pH	2	3	is in range
C: Flow Rate	0.8	1.2	is in range
K_1	1.11	1.39	Minimize
Rt_2	3.09	7.73	Minimize
$Rs_{1,2}$	5.934	9.772	is in range

From the above table it could be seen under the column criteria that the response of Rt2 was minimized to shorten the analysis time and the response of Rs1,2 was minimized to allow the baseline separation of Metformin. To separate the first eluting peak of Dapagliflozin from the solvent front, *k1* was is in range. The importance could range from 1 to 5 which emphasized a target value. Following the conditions and restrictions above,the optimization procedure was carried out. The response surface obtained for the global desirability function was presented in figure 3.

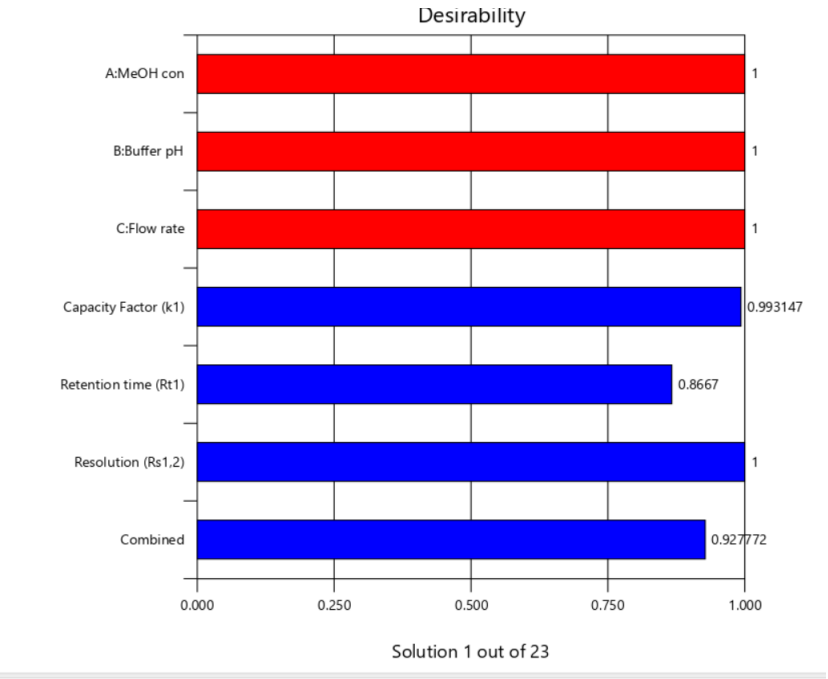
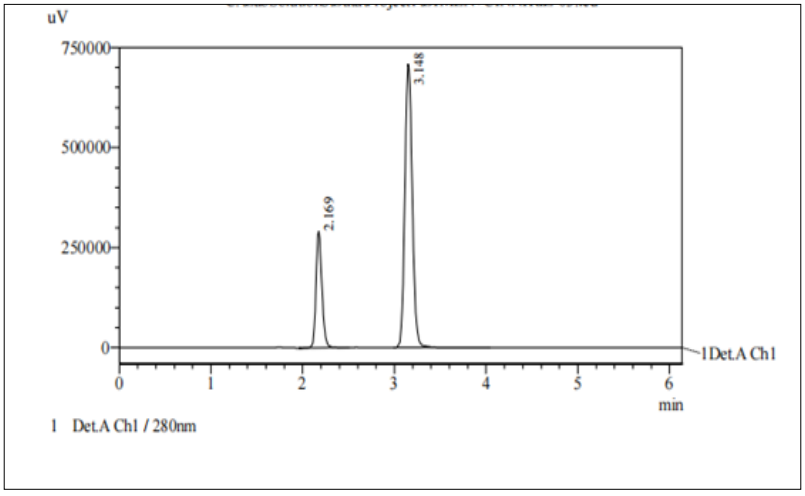


Fig 3 Graphical representation of overall desirability function

From figure 3 it could be concluded that there was a set of coordinates producing a high desirability value (D = 0.9272) were MeOH concentration of 40%, buffer pH of 2.41, and flow rate of 1.024 ml/min. The optimized assay conditions were MeOH: Water buffer (40:40:20%v/v) (pH 2.6) as mobile phase at a flow rate of 1ml/min. and UV detection at 215 nm. The predicted response values corresponding to the later value of Dwere k1 = 1.25, $Rs_{1,2}$= 6.45and Rt2 = 4.25 min. The prediction efficiency of the model was confirmed by experimenting with the optimal condition and the corresponding chromatogram as shown in figure 4. The observed difference between the predicted and experimentalresponses was found to be in good agreement, within a difference of 4.0% was shown in table4.



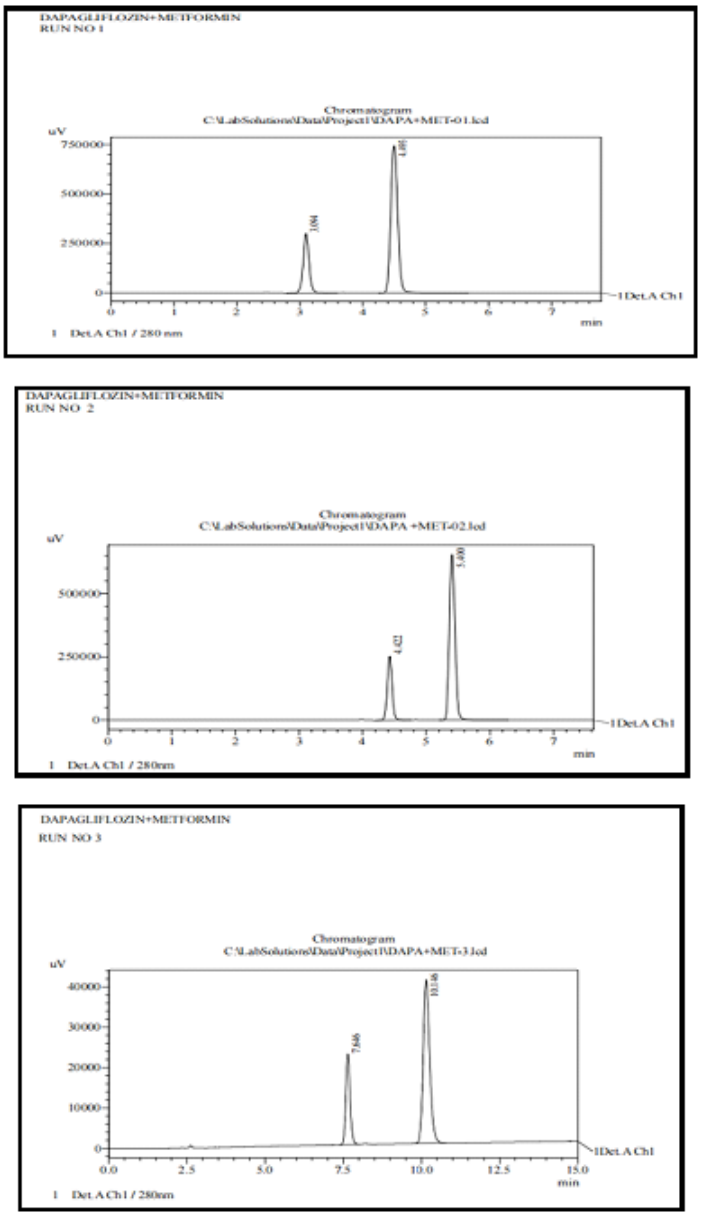


Fig 4 Chromatograms for comparison of Experimental and redictive Value ofdifferent Functions

Table 4 Comparison of Experimental and Predictive values of different functions underOptimal Conditions.

Optimumconditions	**Factors**			**Responses**		
	MeOH conc (%)	**Buffer pH**	**Flow rate ml/min**	**K_1**	**Rt_1**	**$Rs_{1,2}$**
Predictive	55	2.410	1.024	1.112	3.709	6.515
Experimental	55	2.410	1.024	1.122	3.75	6.58
Average error (1-6%)				1.08	1.11	0.99
Desirability value= 0.99						

Table 5 System suitability parameters

Parameters	Compound	
	Dapagliflozin	**Metformin**
Tailing factor	1.19	1.15
Retention time (Rt) in min	2.17	3.15
Theoretical plates (N)	3561.37	5690.11
Resolution (Rs)	-----	6.214

The optimized assay method was specific about the placebo used in this study because there was no excipients peak co-eluted with the analytes. No interferences were observed as shown in figure 10. The method was also selective because there were no interferences observed from any of the excipients in the tablet formulation tested.

Report for validation parameter

Parameter	Dapagliflozin	Metformin
Linearity range (µg/ml)	1-9	50-450
Slope	53216	2910
Intercept	164675	43700
Regression coefficient	0.9998	0.9993
Accuracy %recovery	100.85	100.52
Precision % RSD	0.3711	0.2379
Assay %	99.73	99.87
LOD	0.1530	1.14
LOQ	0.4637	3.46

CONCLUSION

A simple, rapid, and accurate RP-HPLC method was developed for the simultaneous estimation of Dapagliflozin and Metformin in bulk and combined pharmaceutical tablet dosage form using experimental design. Response surface methodology and central composite design were used to find out the optimized assay conditions of Acetonitrile: Methanol: Water buffer (pH 2.6) 40:40:20%v/v as mobile phase at a flow rate of 1 ml/min and UV detection at 215 nm. With the optimized conditions, the drugs were linear with the concentration range of 1 to 9 µg/ml for Dapagliflozin, 50 to 450µg/ml. The correlation coefficients of the Dapagliflozin and Metformin were found to be 0.9998 and 0.9993 respectively. The percentage purity was found to be 99.73 for Dapagliflozin, 99.87 for Metformin. The precision was confirmed by repeating the analysis six times. The accuracy was confirmed by recovery studies. The % recovery was found to be 100.85 for Dapagliflozin and 99.96 for Metformin respectively

Therefore, this proposed HPLC method can be used routinely for the quality control analysis of simultaneous estimation of Dapagliflozin and Metformin in the bulk and pharmaceutical tablet dosage form.

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