BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902019000218129

Article

Application of quality by design approach in RP-HPLC method development for simultaneous estimation of saxagliptin and dapagliflozin in tablet dosage form

Aruna Gundala[1*], KVSRG Prasad[2], Bharathi Koganti[2]

[1]Department of Quality Assurance, Krishna Teja Pharmacy College, Tirupati, Andhra Pradesh, India, [2]Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati, Andhra Pradesh, India.

A simple, sensitive, precise, accurate and robust high performance liquid chromatographic method has been developed for simultaneous estimation of saxagliptin (SAXA) and dapagliflozin (DAPA) in pharmaceutical formulation. Design of experiments (DoE) was applied for multivariate optimization of the experimental conditions of RP-HPLC method. Risk assessment was performed to identify the critical method parameters. Three independent factors; mobile phase composition, flow rate and column temperature were used to design mathematical models. Central composite design (CCD) was used to study the response surface methodology and to study in depth the effects of these independent factors. Desirability function was used to simultaneously optimize the retention time and resolution of SAXA and DAPA. The optimized and predicted data from contour diagram consisted of acetonitrile and ortho phosphoric acid (0.1%) in the ratio of 50:50 respectively, at a flow rate of 0.98 ml/min and column temperature 31.4 °C. Using these optimum conditions baseline separation of both drugs with good resolution and run time of less than 6 min were achieved. The optimized assay conditions were validated according to ICH guidelines. Hence, the results clearly showed that Quality by design approach could be successfully applied to optimize RP-HPLC method for simultaneous estimation of SAXA and DAPA.

Keywords: Saxagliptin/pharmacology. Dapagliflozin/pharmacology. Quality by Design. RP-HPLC/ methods. Optimization/methods. Chromatography, High Pressure Liquid/ methods. Dosage Forms. Tablets/administration & dosage.

INTRODUCTION

Saxagliptin (SAXA), chemically, known as (1s,3s,5s)-2-[(2s)-2-amino-2-(3-hydroxyl-tricyclo[3.3.1.1]dec-1-yl)acetyl]-2-azabicyclo[3.1.0] hexane-3-carbonitrile (Figure 1), is a potent, selective, long-acting, and reversible inhibitor of the enzyme dipeptidyl peptidase 4 (DPP-4) used for treatment of type 2 diabetes mellitus. It is used as monotherapy or in combination with other drugs (Scheeren *et al.*, 2015; Hanan *et al.*, 2017).

Dapagliflozin (DAPA) is chemically described as (1s)-1,5-anhydro-1-C-[4-chloro-3-[(4-ethoxy phenyl) methyl]phenyl]-D-glucitol (Figure 2) (Meira *et al.*, 2017). It belongs to a new class of oral antidiabetic drugs called sodium glucose cotransporter 2 (SGLT2) inhibitors. These sodium glucose cotransporters are responsible for glucose reabsorption in the kidney (Manasa *et al.*, 2014). DAPA is a first generation, selective SGLT inhibitor that blocks glucose transport with 100- fold selectivity for SGLT2 over SGLT1 (Jani, Shah, Kapupara, 2015a). The US FDA has approved a once-daily dose of Qtern (10 mg Dapagliflozin and 5 mg Saxagliptin) for the treatment of type-2 diabetes (Madhavi, Prameela Rani, 2017).

An extensive literature survey has revealed that there is no reverse phase high performance liquid chromatographic (RP HPLC) method available for individual or simultaneous estimation of SAXA and DAPA in bulk, or pharmaceutical dosage forms use an experimental design approach. A few analytical methods were reported in the literature for the determination of SAXA alone. Stability indicating RP HPLC and RP-LC-PDA methods for determination of SAXA in pharmaceutical dosages were developed (Scheeren *et al.*, 2015; Konari, Jacob, 2015). SAXA was estimated,

Correspondance: A. Gundala. Department of Quality Assurance, Krishna Teja Pharmacy College, Tirupathi, A.P, India. Tel: +91-9704624158. E-mail: garuna.mpharm@gmail.com

with other antidiabetic drugs like vildagliptin, using spectrophotometric and spectrofluorimetric methods from bulk and pharmaceutical dosage forms (Marwa, 2013) and also with metformin hydrochloride, using the RP column liquid chromatographic method in binary mixtures (Mohammad, Ehab, Marwa, 2012), The HPLC method is used in active drug and pharmaceutical dosage forms (Hanan *et al.*, 2017) and stability indicating RP HPLC method for the determination of saxagliptin and metformin in bulk and pharmaceutical product (Ramesh, Senthil Kumar, 2016). Some analytical procedures have been reported for quantitative determination of DAPA alone by UV spectrophotometry only in API (Manasa *et al.*, 2014) and the RP HPLC method in bulk and tablet dosage forms (Subrata, Vipul, 2017). Simultaneous estimation of DAPA and metformin hydrochloride has also been developed using the UV spectrophotometric method and first derivative photometric method in a synthetic mixture (Jani, Shah, Kapupara, 2015b). Literature has depicted very critical care and typical reports of RP HPLC for simultaneous estimation of SAXA and DAPA in bulk and tablet form namely, a new stability indicating by the RP HPLC method (Vinutha, Chowdary, Prasad, 2017) and precise UPLC (Madhavi, Prameela Rani, 2017). These methods did not describe the design space as per recent FDA guidelines. The use of 'Quality by Design' (QbD) or 'Design of Experiments' (DoE) is recommended to achieve robustness during analytical method validation by statistical quality control monitoring and by the study of factors that negatively affect quality in pharmaceutical analysis (Ganorkar, Dhumal, Shirkhedkar, 2017).

The traditional approach of method development is comprised of trial and error and by varying one factor at a time (Yadav *et al.*, 2016).This approach often encounters difficulties in optimizing robust chromatographic conditions due to various factors, like limited availability of the chromatographic column, solvents, chemicals, and critical physicochemical properties of the analyte. Recently the FDA has approved few new drug applications (NDA) that have applied the QbD approach to analytical techniques, like HPLC and UV Spectrophotometry, in which regulatory flexibility has been granted for movement within the defined method operable design region (MODR) (Peraman *et al.*, 2015). Since its implementation by the FDA, QbD has been an integral part of the pharmaceutical product development process, impacting its robustness (Awotwe-Otoo *et al.,* 2012). A modern QbD based treatment of the robustness of the HPLC method requires the assessment of all factors which most strongly influence the results of the method. The experimental verification of many factors simultaneously is impractical and associated with difficulties and more expense. To overcome the challenge and reduce the experimental workload, a thorough understanding of the response of the system quality to system parameters that leads ultimately to the establishment of design space for the method is important (Awotwe-Otoo *et al.*, 2012; Monks *et al.*, 2012).

Hence, our quest for developing a simple, rapid, precise, robust RP HPLC method for analysis of SAXA and DAPA, assisted with DoE, was developed and central composite design (CCD) for evaluation of robustness of developed method, followed by graphical interpretation of data by response surface methodology (RSM).

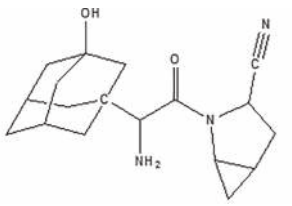


FIGURE 1 - Chemical structure of saxagliptin.

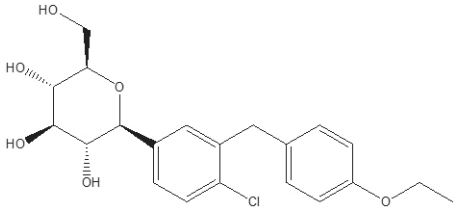


FIGURE 2 - Chemical structure of dapagliflozin.

MATERIAL AND METHODS

Material

Pure SAXA and DAPA samples were obtained from Spectrum Pharma Research Solutions, Hyderabad, India as a gift. Acetonitrile was of HPLC grade and orthophosphoric acid was of analytical-reagent grade supplied by Merck (Mumbai, India). HPLC grade water was obtained following distillation in glass and passage through a Milli-Q Academic system (Millipore, Bangalore, India) and was used to prepare all solutions. Qtern tablet formulation was purchased from local market. This formulation had a label claim of 5 mg of SAXA and 10 mg of DAPA each.

Instrumentation

A Waters HPLC instrument- LC-20AD (Japan) equipped with rheodyne 7725 injection valve with a

20 µL loop volume and binary gradient pump was used. The system also included a PDA (Shimadzu, SPD-20A) detector operated at a wavelength of 210 nm. Data were acquired and processed by using empower 2 software. Chromatographic separation was performed using Discovery C18 column (250 mm, 4.6 mm, and 5 µm).

Software

Experimental design (CCD), desirability function, and data analysis calculations were performed by using Design-Expert version 10.0.3.1.

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. The solution was degased by sonication and then brought up to the volume (1000 mL) with water.

Preparation of standard stock solution

About 5 mg of SAXA 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 SAXA 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.

Software aided method optimization

Literature revealed that some design methodologies were presented to assess the robustness of method (Ganorkar, Dhumal, Shirkhedkar, 2017). They applied in the circumstances of optimizing separation techniques during screening, testing of robustness and also in the context of optimizing formulation, products, or method. Here in the present work, the important chromatographic factors were selected, based on preliminary experiments and prior knowledge from the literature. A good choice among the screening design in the testing of few factors (three or less) for robustness, may be CCD because of its efficiency, with respect to number of runs required (Petkovska, Cornett, Dimitrovska, 2008).Various factors were considered for method development, including volume of organic solvents in the mobile phase, buffer, flow rate, and column temperature (Dawud, Shakya, 2014). Thus, CCD was employed to evaluate the effects of three independent chromatographic parameters on the three defined key response variables. The design was comprised of 20 experimental runs and helped in screening of factors by evaluating their main effect to get outcomes of the study. A 3^2 factorial design indicated that there were two levels and three factors were involved. The two levels were low (-1) and high (+1), whereas factors were (X_1) proportion of organic solvent used in mobile phase (45% and 55%), (X_2) flow rate of mobile phase (0.9 and 1.1 mL/min), and (X_3) column temperature (28 and 32 °C) . The retention time of SAXA (Y_1), retention time of DAPA (Y_2), and resolution (Y_3) were used as responses in experimental design and were shown in Table I. The resulting data were fitted into Design-Expert version 10.0.3.1. Response surface quadratic methodology was a suitable method and was used to explore, to investigate behavior of the response around optimized values of the factors, and to attain the best system performance (Ficarra *et al.*, 2002). Analysis of variance (ANOVA) was applied to examine the significance of the model. From this optimized method, conditions were selected and subjected to verification for method performance, like accuracy, precision (less than 2% RSD), and robustness as targeted

response (Peraman *et al.*, 2015). Twenty experiments were constructed using the conditions and observed responses are described in Table II.

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. (Ganorkar, Dhumal,

TABLE I - Experimental plan of CCD showing factors with levels

Factors	Code	Range levels	
		Low (-1)	High (+1)
Proportion of organic solvent used in mobile phase	X_1	45	55
Flow rate of mobile phase (mL/min)	X_2	0.9	1.1
Column temperature	X_3	28	32
Responses			
Retention time of SAXA	Y_1	-	-
Retention time of DAPA	Y_2	-	-
Resolution	Y_3	-	-

TABLE II - Coded values for factor level and observed responses in CCD for 20 analytical trials

Experiment (Run)	Type	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	Factorial	45	0.9	28	3.204	3.876	3.5
2	Axial	41.591	1	30	2.889	3.575	3.7
3	Factorial	45	1.1	32	2.582	3.17	3.8
4	Axial	58.409	1	30	2.687	3.297	3.8
5	Center	50	1	30	2.771	3.405	3.6
6	Center	50	1	30	2.772	3.407	3.6
7	Center	50	1	30	2.771	3.406	3.6
8	Axial	50	1	26.6364	2.769	3.746	4.8
9	Center	50	1	30	2.772	3.407	3.6
10	Factorial	55	1.1	32	2.491	3.05	4.2
11	Axial	50	1.16818	30	2.395	2.948	3.7
12	Center	50	1	30	2.77	3.406	3.6
13	Factorial	55	1.1	28	2.486	3.157	3.8
14	Factorial	55	0.9	28	3.046	3.833	4.1
15	Center	50	1	30	2.771	3.407	3.6
16	Factorial	45	1.1	28	2.615	3.517	4.7
17	Axial	50	0.831821	30	3.33	4.107	3.8
18	Factorial	55	0.9	32	3.029	3.716	4.3
19	Axial	50	1	33.3636	2.792	3.42	4.3
20	Factorial	45	0.9	32	3.17	3.903	4.2

Shirkhedkar, 2017). 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 SAX 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 (Thakur, Kaur, Sharma, 2017; Agrawal, Desai, Jani, 2016).

Linearity

Linearity of the developed method was established at six levels over the range of 12.5-75 µg/mL for SAXA and 25-150 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.

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 (Patel *et al.*, 2017). 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 (Pradipbhai *et al.*, 2017). Six homogenous samples of SAXA and DAPA were assayed to assess the method precision (Yadav *et al.*, 2016; Thakur, Kaur, Sharma, 2017).

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

LOD and LOQ of SAXA 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.

RESULTS AND DISCUSSION

Preliminary studies and factor selection

There were only a few works reported on implementation of QbD in analytical method development (Peraman *et al.*, 2015).

In search of a simple, robust, and cost effective RP-HPLC method for estimation of SAXA and DAPA in tablet formulation, a preliminary study was initially carried out. The important chromatographic factors were selected, based on preliminary experiments and prior reports from the literature. Such investigations to select the factor levels for screening and optimization studies revealed that mobile phase conditions needed to be optimized so that both SAXA and DAPA would be separated in a short run time. Mobile phase composition of 0.1% OPA buffer and acetonitrile was found to be more suitable for the simultaneous estimation of both the drugs and the volume of acetonitrile resulted in a large change in retention time. Hence, it is considered as one of the critical parameters for method development.

For further study, different reverse phase columns were tried but finally satisfactory separation was obtained on a Discovery C18 column. From Ishikawa diagram and Pareto ranking analysis, preliminary experiments were conducted and the critical parameters selected for further study, were flow rate and column temperature which were found to have the most influential effect on system suitability parameters. Studies carried out with protamine sulphate also resulted in selection of similar critical parameters (Awotwe-Otoo *et al.*, 2012).

QbD assisted method development

CCD design was employed in the present analytical method optimization study. It is an efficient and comprehensive experimental design based on systematic scouting of three key components of the RP-HPLC method (volume of organic phase, flow rate, and column temperature) is depicted.

For the RP-HPLC method, a multivariate approach DoE with CCD was applied to study the simultaneous variations of the factors on considered responses, such as retention time of SAXA (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 CCD 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 SAXA (Y_1) and DAPA (Y_2) were in reasonable agreement with adjusted R- squared values i.e., the difference is less than 0.2, as reported by other authors (Pradipbhai *et al.*, 2017). A negative predicted R- squared value for resolution (Y_3) 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 Y_1, Y_2, and Y_3 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 SAXA (Y_1), retention time of DAPA (Y_2), and resolution (Y_3) 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 Y_1 and Y2, while a 1.44% chance for resolution (Y_3) 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 Y_1, Y_2, and Y_3 are:

$$SAXA\ (Y_1) = +2.77 - 0.063X_1 - 0.28X_2 - 2.95X_3 + 9.87X_1X_2 + 6.87X_1X_3 + 2.87X_2X_3 + 9.15X_1^2 + 0.03X_2^2 + 6.50X_3^2;$$

$$DAPA:\ (Y_2) = +3.41 - 0.08X_1 - 0.32X_2 - 0.08X_3 - 0.03X_1X_2 + 0.01X_1X_3 - 0.04X_2X_3 + 0.01X_1^2 + 0.044X_1^2 + 0.06X_3^2;$$

$$Resolution:\ (Y_3) = +3.60 + 0.02X_1 + 0.01X_2 - 0.03X_3 - 0.15X_1X_2 + 0.10X_1X_3 - 0.17X_2X_3 + 0.06X_1^2 + 0.06X_2^2 + 0.34X_3^2.$$

As per the values of coefficient from the above equations and their signs, it is clear that factors, such as mobile phase composition (X_1), flow rate (X_2), and column temperature (X_3), had a negative effect on retention time of SAXA and DAPA, Y_1 and Y_2. The column temperature (X_3) had a negative effect on resolution (Y_3), whereas mobile phase (X_1) and flow rate (X_2) had positive effects. Interactions of X_1 and X_2 had a positive effect on Y_1 and Y_2 and a negative effect on Y_3; X_2 and X_3 had a positive effect on Y_1 and a negative effect on Y_2 and Y_3; X_1 and X_3 had a positive effect on Y_1 and Y_2 and a negative effect on Y_3. The squares of factors, X_1^2, X_2^2, and X_3^2, 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 (Awotwe-Otoo *et al.*, 2012). The contour plots showed curvature, displaying a nonlinear effect of factors on responses. Figures 3 and 4 showed 2D (A) and 3D (B) contour plots displaying the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of SAXA (Y_1) and DAPA (Y_2). A curvilinear increasing trend was observed for the mobile phase ratio (X_1) and flow rate (X_2), which showed higher resolution time of SAXA (Y_1), as well as DAPA (Y_2) at lower levels. Therefore, lower levels of X_1 and X_2 were recommended to achieve high retention time of SAXA (Y_1) and DAPA (Y_2). The study of 3D and 2D contour plots presented in Figure 5 showed curvature effects of the mobile phase ratio (X_1) and flow rate (X_2) on resolution. An increasing curvature trend was observed for both X_1 and X_2, which showed higher resolution at higher levels. Therefore, optimized levels of X_1 and X_2 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 (Costa, Lourenço, Pereira, 2011), 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 (X_1) buffer and acetonitrile 50:50, flow rate (0.96 mL/min), and column temperature 31.14 °C which resulted in retention time of SAXA (Y_1) 2.79±0.0162, retention time of DAPA (Y_2) 3.45±0.013, and resolution (Y1) 3.7±0.002 min, respectively, as shown in Figure 7.

Method validation

According to the USP, system suitability tests are

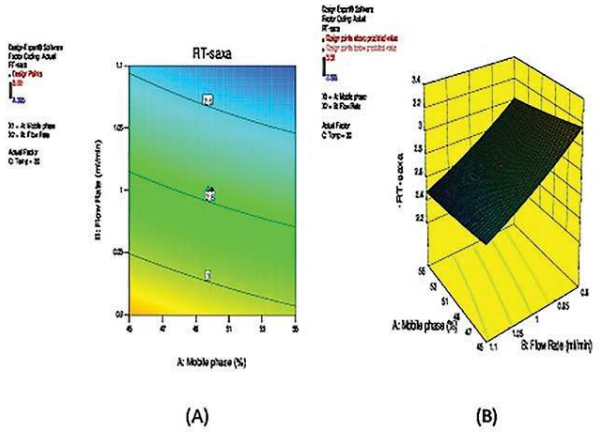


FIGURE 3 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of SAXA (Y_1).

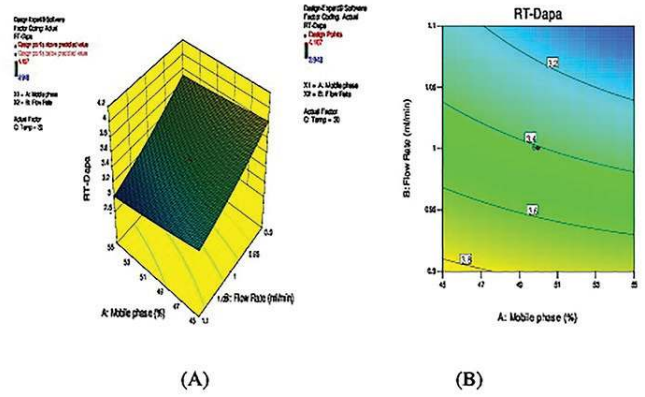


FIGURE 4 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of DAPA (Y_2).

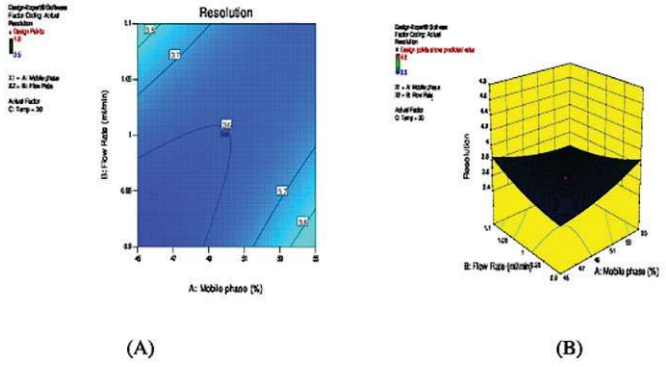


FIGURE 5 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on resolution (Y_3).

an integral part of liquid chromatographic methods. The column efficiency, as determined from number of theoretical plates for both the drugs, was found to be more than 4000, resolution was 3.4, and tailing was found to be less than

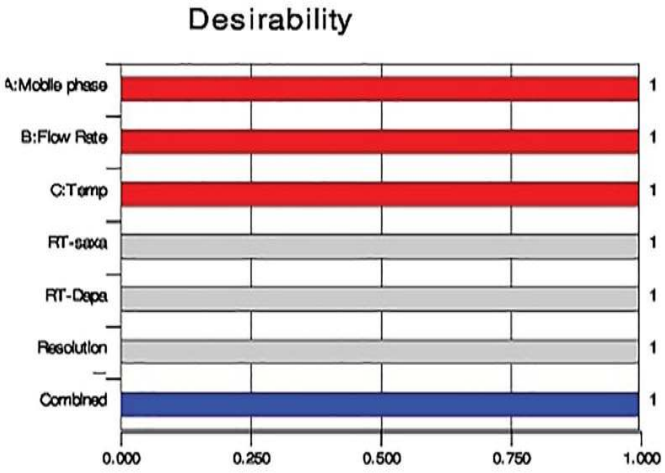
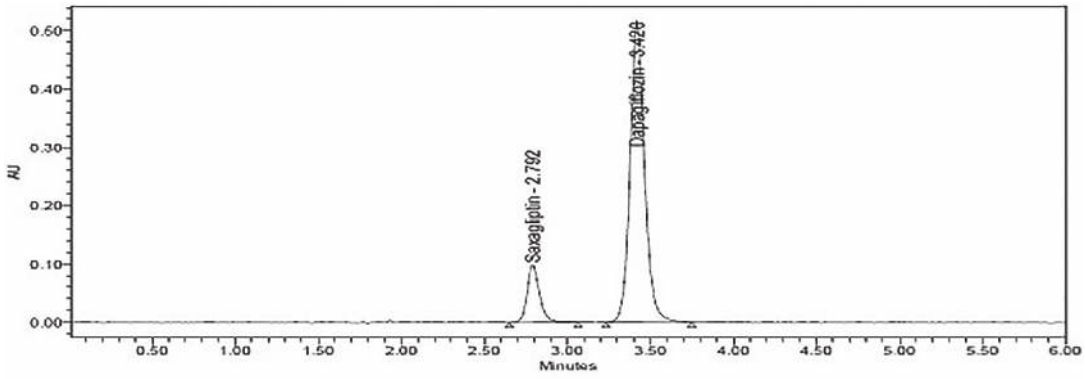


FIGURE 6 - Desirability function representation basis unity=1.

2. The percent relative standard deviation for six replicate injections was found to be 1.2 in the given concentration of 5µg/mL for SAXA and 0.3 in the given concentration of 10µg/mL for DAPA, respectively. As % RSD was found to be less than 2%, it has shown good injection repeatability (Thakur, Kaur, Sharma, 2017). Linearity of the developed method was confirmed by plotting the linearity curve over concentrations ranging from 12.5-75 µg/mL for SAXA and 25-150 µg/mL for DAPA, with a correlation coefficient (r^2=0.999) for both the drugs, shown in Table IV. The obtained correlation coefficient (r^2=0.999) demonstrates excellent correlation between peak area and concentration. For the recovery study, different concentrations of samples (50, 100, and 150%) of standard concentrations for both drugs were prepared and showed recovery of 99.91±1.10 % and 98.92 ±0.7997 % for SAXA and DAPA, respectively. Data is shown in Table IV, indicating that the developed method has high level of accuracy with % RSD 0.27 and 0.4 for SAXA and DAPA, respectively. Intermediate precision and repeatability were carried out and the resultant data are given in Table IV. The precision values for both drugs were less than 2%., indicating that the method was repeatable and precise (Dawud, Shakya, 2014). The LOD and LOQ were found to be 0.13 and 0.39 µg/mL, respectively for SAXA, and 0.09 and 0.27 µg/mL, respectively for DAPA. The insensitivity of the RP-HPLC method to minor changes in the optimized experimental changes was demonstrated by its robustness to such slight changes. The mobile phase composition, flow rate, and column temperature caused significant effects in the retention time of SAXA and DAPA, as well as resolution.

CONCLUSION

The present study entails systematic QbD, based



	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	Asym @ 10
1	Saxagliptin	2.792	513372	14.21	7381		1.3
2	Dapagliflozin	3.420	3098582	85.79	7626	4.3	1.2

FIGURE 7 - Optimized RP-HPLC chromatogram for SAXA and DAPA at 210 nm

TABLE III - ANOVA regression analysis for models and responses

Response	Mean	SD[a]	%CV[b]	Press Value	R^2 [c]	Adjusted R^2	Predicted R^2	Adequate Precision	SS[d]	d[e]	MS[f]	F[g]	P
Retention time of SAXA (Y_1)	2.81	0.014	0.50	0.015	0.9983	0.9968	0.9873	96.445	1.16	9	0.13	666.4	0.0001
Retention time of DAPA ($Y_{2)}$	3.49	0.052	1.48	0.23	0.9845	0.9705	0.8653	29.953	1.70	9	0.19	70.44	0.0001
Resolution (Y_3)	3.91	0.24	6.05	4.89	0.8003	0.6205	-0.7445	6.118	2.25	9	0.25	4.45	0.0144

[a] Standard deviation, [b] Coefficient of variations, [c] Coefficient of Regression, [d] Sum of squares, [e] Degrees of freedom, [f] Mean sum of squares, [g] Fischer's ratio

TABLE IV - Validation results for SAXA and DAPA

PARAMETERS		SAXA	DAPA
System suitability parameters			
No. of theoretical plates	Mean ± SD*	4613.66±96.70	4599±85.082
	% RSD	2.0	1.8
Resolution	Mean ± SD	-	3.266±0.057
	% RSD	-	1.7
Tailing factor	Mean ± SD*	1.44±0.005	1.60±0.015
	% RSD	0.3	0.9
Linearity			
Range(µg/ml)		12.5-75	25-150
Slope		11316	38865
Intercept		3931.8	5685.8
Correlation coefficient		0.999	0.999
Accuracy			
Recovery studies		99.91±1.10	98.92±0.7997
%RSD**		1.10	0.87
Precision			
Repeatability	% RSD*	0.27	0.4
Intermediate precision	% RSD*	0.3	0.6
LOD (µg/mL)		0.13	0.09
LOQ (µg/mL)		0.39	0.27

*Mean of six determinations, **Set of three determinations

development of a simple, rapid, precise, and cost effective RP-HPLC method for simultaneous estimation of SAXA and DAPA, for the first time. 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 SAXA and DAPA. CCD was applied to optimize the resolution as response between SAXA and DAPA in a relatively short time (6 min). In the optimized model, the acetonitrile and orthophosphoric acid (0.1%) in the ratio of 50:50 indicates the suitability for estimation of SAXA and DAPA. The flow rate of the mobile phase was optimized at 0.98 ml/min and column temperature at 31.4 °C respectively. The validation study supported the selection of the best conditions by confirming that the method was selective, specific, accurate, linear, precise, and robust. Therefore utilization of the response surface technique provides a better insight for method development and robustness testing. This developed method satisfies the design space concept and is suitable for regulatory submission under regulatory flexibility.

ACKNOWLEDGEMENT

The author would like to thank to spectrum labs, Hyderabad for providing gift samples of Saxagliptin, Dapaglifazoin.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest regarding this manuscript.

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Received for publication on 07th March 2018
Accepted for publication on 27th June 2018