Response surface methodology in Spectrophotometric Estimation of Saxagliptin, Derivatization with MBTH and Ninhydrin

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INTRODUCTION

Saxagliptin (Onglyza) is an oral antihyperglycemic drug belonging to the dipeptidyl peptidase-4 inhibitor class.¹ Saxagliptin is indicated for patients with type 2 diabetes to improve glycemic control, used alone or in combination with metformin and/or insulin when these drugs do not provide adequate glycemic control. The recommended dose of saxagliptin is 2.5 mg/5 mg once daily.²,³ Few methods were reported in the literature for the analysis of saxagliptin using UV-spectrophotometric,⁴-¹¹ spectrofluorometric, HPTLC¹² and HPLC¹³,¹⁴ techniques. Colorimetric methods are relatively simple, faster (in terms of sample preparation), inexpensive than chromatographic techniques, and more sensitive, specific over UV-spectrophotometry due to selective chemical reaction of analyte with reagent to yield a colored derivative.¹⁵-¹⁹ The literature review revealed, two colorimetric reports²⁰,²¹ for estimation of saxagliptin using chromogenic reagents (DDQ {2,3-dichloro-5,6-dicyano-1,4-benzoquinone}; TCNQ {7,7,8,8-tetracyanoquinodimethane}; NQS {1,2-naphthoquinone-4-sulfonic acid}; NBD-Cl {4-chloro-7-nitrobenzofurazan}). There was no method developed using proposed chromogenic agents such as MBTH (3-Methyl-2-benzothiazolinone hydrazone), ninhydrin which can produce more sensitive and specific methods for the analysis of saxagliptin.

The literature methods utilized conventional strategy in experimentation i.e. varying one factor at a time (OFAT), which may deliver ambiguous and inept optimization in analytical method development, so it must be avoided. This necessitating the use of systematic and statistical approach for optimization of method variables to attain consistent results,²²,²³ which can be achieved through analytical quality by design (AQbD) strategy. The AQbD in method development facilitates the simultaneous evaluation of significant variables through design of experiments (DoE) and response surface analysis for accomplishing enhanced method performance.²⁴,²⁵ The contemplated research aimed to exploit AQbD approach in the development of visible-spectrophotometric method for estimation of the saxagliptin through chemical derivatization technique.

MATERIALS AND METHODS

Instrumentation and Chemicals

Double beam 1800 UV-Visible spectrophotometer (Shimadzu, Japan), analytical balance (Shimadzu AUX 220, Japan) and ultrasonic cleaner (Sonica) were used for the study. Ethanol was purchased from Qualigens, Mumbai. Ferric chloride, sodium hydroxide, MBTH and ninhydrin were purchased from SD Fine-Chem Ltd., Mumbai. Double distilled water was used throughout the study. Saxagliptin standard gift sample was provided by Hetero Laboratories Pvt Ltd., Hyderabad, India. Marketed dosage form (Onglyza®, AstraZeneca) of saxagliptin was procured from the local pharmacy.

Experimental design, data analysis and response surface plots were developed employing Design-Expert trial version 11.0.5.0 software (Stat-Ease Inc. Minneapolis).

Preparation of solutions

The standard stock solution (100 µg/mL) of saxagliptin was prepared by solubilizing accurately weighed 10 mg of saxagliptin in 10 mL of ethanol and diluted to 100 mL with distilled water. Working standard solution-1 was prepared by diluting 1 mL standard stock solution to 10 mL with distilled water to get 10 µg/mL of saxagliptin.
Working standard solution-2 was prepared by diluting 0.1 mL working standard solution-1 to 10 mL with distilled water to get 0.1 µg/mL of saxagliptin.

Solutions of MBTH reagent (1.1 % w/v), ferric chloride (3.5 % w/v), ninhydrin (1 % w/v) and sodium hydroxide (0.4 M) were prepared in distilled water.

**Experimental design and optimization**

**Method-1:** Box-Behnken design (BBD) with response surface methodology (RSM) was employed to optimize reaction (saxagliptin-MBTH) conditions for colorimetric method development. The effect of three method variables (at high, low levels) such as concentration of MBTH (A1): 0.8, 1.2 %w/v; concentration of ferric chloride (B1): 1.5 %w/v; reaction time (C1): 10 - 30 min; on the response (Y1) i.e. absorbance of bluish-green coloured complex was studied at 600 nm in spectrophotometer. Randomized order in experimentation was followed to abate the bias effects of uncontrolled variables for 17 experimental runs under BBD using working standard solution-2 (0.1 µg/mL).

**Method-2:** Central composite design (CCD) with RSM was exploited to optimize experimental conditions for colorimetric estimation of saxagliptin, upon derivatization with ninhydrin reagent. Working standard solution-1 (10 µg/mL) was utilized for experimentation. The present method optimization was premeditated with 15 experimental runs under CCD (4 factorial points, 5 center points and 6 axial points) to study the influence and interaction of three method parameters (at high, low levels) namely, concentration of ninhydrin reagent (A2): 0.8, 1.2 %w/v; concentration of sodium hydroxide (B2): 0.2, 0.6 M; heating time (C2): 5 - 15 min on the response (Y2) i.e. absorbance of Ruhemann’s purple, measured at 585 nm in spectrophotometer. Statistical analysis was performed together with experimental design (BBD/CCD) in Design-Expert software. The significance of variables was studied through (p-values) analysis of variance (ANOVA). Multiple regression analysis was performed and estimated the correlation coefficient (r²) for response studied. The main and interaction effects of variables were detected via best-fitted models, selected based on various parameters like PRESS (predicted error sum of squares), R² (adjusted, predicted), % CV, adequate precision and lack of fit analysis. Contour plots (2D) and response surface plots (3D) were employed for quantitative identification of influence of each variable (along with interaction) on the response (Y). The design space was generated as a multi-dimensional combination between variables and response for the maximum desirability function.

**Validation of methods**

The proposed methods were validated for linearity, accuracy, precision, LOD (limit of detection), and LOQ (limit of quantification) as per ICH (international conference on Harmonization) guidelines.

**Linearity**

**Method-1:** Aliquots (0.01, 0.05, 0.1, 0.15 ,0.2, 0.25 mL) of standard solution (10 µg/mL) of saxagliptin were taken into a series of 10 mL volumetric flasks. To these 0.5 mL of 1.1 % MBTH solution and 0.5 mL of 3.5 % FeCl₃ were added, shaken vigorously and kept a side for 20 min. Then volume was then made up to the mark with water to prepare a series of standard solutions containing 0.01 - 0.25 μg/mL of saxagliptin. Then the absorbance of the blue-green colored chromogen was measured at 600 nm against corresponding reagent blank. The amount of saxagliptin was computed from the Beer – Lambert’s plot.

**Method-2:** Aliquots (0.1, 0.2, 0.3, 0.6, 0.8, 1 mL) of standard drug solution (100 µg/mL) of saxagliptin were taken into a series of 10 mL volumetric flasks. To these 2 mL of 1 % ninhydrin reagent and 2 mL of 0.4 M NaOH were added, shaken vigorously and heated for 13 min. The volume was then made up to the mark with water to prepare a series of standard solutions containing 1 - 10 µg/mL of saxagliptin. Then the absorbance of the yellow-purple colored chromogen was measured at 585 nm against corresponding reagent blank. The amount of saxagliptin was computed from the Beer – Lambert’s plot.

**Precision**

The intra-day and inter-day precision of the proposed colorimetric methods were determined for three different concentrations of saxagliptin within the linearity range. Estimated the corresponding response of solutions prepared three times on the same day and three different days of over a week. The results were reported in terms of relative standard deviation (% RSD).

**Accuracy**

The accuracy of the method was determined by calculating recoveries of saxagliptin by the method of standard additions. Known concentration of saxagliptin solutions were added at 80, 100 and 120% levels to pre-quantified sample solutions of saxagliptin and analyzed through proposed methods. Each sample was prepared in triplicate at each level. The amount of saxagliptin was estimated by applying obtained values to regression equation.

**Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) of saxagliptin by proposed methods were derived from the standard calibration curve using 3.3 σ/s and 10 σ/s, formulae respectively, where s is the slope of the calibration curve and σ is standard deviation of the y-intercept of the regression equation.

**Assay of saxagliptin marketed dosage form**
Twenty tablets of saxagliptin (Onglyza®) were weighed and powdered. The powder quantity equivalent to 10 mg of drug was dissolved in 10 mL ethanol, filtered (using Whatman’s filter paper) into 100 mL volumetric flask and made the volume up to the mark with distilled water. From this 1 mL was transferred into a 10 mL volumetric flask, added 2 mL of 1 % ninhydrin reagent and 2 mL of 0.4 M sodium hydroxide, shaken vigorously and heated for 13 min (method-2). For method-1, further dilution of filtrate (1 mL diluted to 10 mL with water) was done. To this 0.1 mL of solution, added 0.5 mL each of 1.1 % MBTH and 3.5 % FeCl3, shaken vigorously and kept aside for 20 min. The volume was made up to the mark with distilled water. The absorbance of the resulting colored solution was measured at 600 (method-1)/ 585 nm (method-2) against the corresponding reagent blank. The amount of saxagliptin was calculated from the Beer – Lambert’s plot.

**Results and Discussion**

**Basis for color development**

Direct spectroscopy method for analysis of a saxagliptin in pharmaceutical formulation may prone interferences due to matrix effect. Chemical derivatization of drug enhances selectivity and sensitivity of the method. The proposed methods subjugated the saxagliptin to chemical derivatization using chromogenic reagents (MBTH and ninhydrin) for its visible spectroscopic analysis.

In Method-1, saxagliptin undergoes an oxidative coupling reaction with MBTH in the presence of oxidizing agent (ferric chloride) to produce bluish-green colored chromogen. Initially, oxidation (loss of 2 electrons and 1 proton) of MBTH by ferric chloride occurs to give an electrophilic intermediate, which coupled with the most nucleophilic site of saxagliptin and forms a Schiff base. This Schiff base upon condensation of an intermediate amine with another molecule of ninhydrin (Figure 2), exhibiting absorption maxima at 585 nm in a spectrophotometer.

In Method-2, ninhydrin undergoes automerism (with loss of water) to 1,2,3-indane trione, which actively react with primary amine group of saxagliptin and forms a Schiff base. In the reaction attack of nucleophile (amine) on the electrophile (carbonyl group) of 1,2,3-indane trione followed by dehydration takes place. Further hydrolysis gives an intermediate amine due to isolation of carbonyl compound. A deep blue/purple color compound (diketohydrindylidene-diketohydrindamine) extensively known as Ruhemann’s purple produces upon condensation of an intermediate amine with another molecule of ninhydrin (Figure 2), exhibiting absorption maxima at 585 nm in a spectrophotometer.

**Evidence of chemical derivatization**

Saxagliptin chemical derivatization with proposed reagents was evidenced by the TLC (Thin layer chromatography) analysis of reaction mixture. Pre-coated TLC plates were spotted separately for method-1 and 2 with a freshly prepared solution of saxagliptin, reagent blank solution and chromogen produced by that method. Plates were developed in saturated chromatographic tanks using ethyl acetate: acetonitrile (7:3) as a mobile phase and spots were visualized in the UV chamber at 254 nm. Three spots were observed with different Rf (Retention factor) values on both plates (method-1 and method-2), indicates the presence of three different compounds. Higher retardation factor values were observed for derivatives (S1), denotes the formation of a new compound by the proposed reaction mechanism.

**Method optimization via AQbD approach**

Optimization of reagent concentration, diluting solvent and time for color development was established to accomplish maximum absorbance and stability. The influence of variables on absorption values of colored species was studied by multivariate approach.

The matrix of BBD, CCD and results of experimental runs performed on UV-Vis spectrophotometer are provided in Table 1. Analysis of variance (ANOVA) was performed for experimental observations to evaluate the significant effect of variables on response, summarized in Table 2. The experimental results are fitted to a second-order polynomial (quadratic) model for the studied response Y (absorbance), given by following equations:

\[
Y_1 = 0.5870 +0.0426 A_1 +0.0413 B_1 +0.0106 C_1 +0.0032 A_1B_1 +0.0040 A_1C_1 +0.0062 B_1C_1 -0.0390 A_1^2-0.0593 B_1^2-0.0190 C_1^2
\]

\[
Y_2 = +0.9806 +0.0788 A_2 +0.0198 B_2 +0.0433 C_2 -0.0444 A_2B_2 +0.0438 A_2C_2 +0.0433 B_2C_2 -0.0726 A_2^2 -0.0824 B_2^2 -0.0379 C_2^2
\]

Statistical analysis data of both methods reveals the significant effect of three variables (A, B, C) on the studied response as their p-values (probability) found below the considered value (p<0.05) at 95 % confidence levels. The high values of regression co-efficient (r²=0.999) obtained, indicates the good correlation between the experimental data and the selected models. Coefficient of variation (CV) found to be less than 5%, indicates reproducibility of model. The model aptness is recognized by the values of low PRESS, high adequate precision (> 4), agreement between predicted and adjusted R² (difference<0.2).

Sensitiveness of a specific response by perturbation of an individual factor from its reference value (while other factors kept constant) was studied through construction of perturbation plots. Steepest slope of perturbation plots (S5-a) observed, indicates that the response (absorbance) in Method-1 is highly influenced by the concentration of MBTH (A1) followed by concentration of ferric chloride (B1). Curvature in plots (S5-b) indicate the response (absorbance) in Method-2 is highly affected by the concentration of ninhydrin (A2) and followed by reaction...
heating time (C2). Variable-response relationship was visualized through contour (2D) and response surface (3D) graphs. These plots can be used to find the response for a given set of input variables. The non-linear trend in response surfaces (S6, S7) was observed, reveals the existence of a high degree of interaction (among variables) that affects the method response. Optimization of variables for maximum spectrophotometric absorbance at respective wavelengths was carried using Derringer's desirability function. The solution with maximum desirability value is selected as an optimized one, out of different solutions provided by the software. The design space generated was portrayed in Figure 3, indicated high performance owing to maximum desirability value (equal to 1) for both methods and unveiled method operable design region with location of optimized solution for the design studied.

The optimized conditions interpreted from statistical and response surface analysis are as follows. Method-1: 1.1 %w/v MBTH (A1), 3.5 %w/v Ferric chloride (B1) and reaction time 20 min (C1). Method-2: 1 %w/v ninhydrin reagent (A2), 0.4 M sodium hydroxide (B2) and reaction heating time 13 min (C2). These reaction conditions are adequate for reproducible, maximum color development for spectroscopic estimation of saxagliptin and were verified through practical experimentation. The UV-Visible absorbance spectrum of saxagliptin under optimized conditions was recorded (S8) for method-1 and 2.

Effect of solvents
Diluting solvent plays an important role in the stability of the colored complex. The effect of different diluting solvents like acetone, acetonitrile, ethanol, methanol, and distilled water have been studied (Figure 4) for measurement under optimized reaction conditions. The best sensitivity, maximum UV absorption, and product stability were attained when water was used as a solvent for both the methods. Both reagents were freely soluble in water. Hence distilled water was selected as a diluting solvent for proposed methods that minimise the cost of experiment and considered as a green approach for spectrophotometric method development.

Stoichiometry of reaction
Stoichiometry of reaction in method-1 and method-2 was studied by Job's continuous variation method. Equimolar solutions (3.174 X 10^-6 M) of saxagliptin and reagents (MBTH and ninhydrin) were prepared in distilled water. The drug and reagent (MBTH: method-1 / ninhydrin: method-2) were mixed in various proportions to produce different mole ratio values (0, 0.2, 0.4, 0.5, 0.6, 0.8, 1). These solutions were analyzed through proposed methods. The stoichiometric relationship displayed in Figure 5. A mole ratio of 0.5 gave the highest absorbance value for method-1 whereas 0.7 for method-2. This indicates that saxagliptin has one center (primary amino group) available for chromogenic reaction with MBTH (1 molecule) and ninhydrin (2 molecules) reagents at their optimum wavelengths.

Validation of proposed methods
The proposed methods were statistically validated as per ICH guidelines and results were depicted in Table 3. Linear regression analysis was performed for the Beer's Law data (S2), and calibration plots (S9) were drawn (correlation co-efficient 0.999). A linear increase in the absorbance was found with an increase in saxagliptin concentration at a range of 0.01 - 0.25 μg/mL and 1 – 10 μg/mL of saxagliptin by method-1 and 2 respectively. Overlaid UV-Visible spectra of saxagliptin in the linearity range was shown in Figure 6. The reproducibility of proposed methods was evinced by precision studies (S3), where no significant difference between intra and inter-day precision values was observed and % RSD values were less than 2. The % recoveries of saxagliptin (S4) denote the fair accuracy of proposed methods with no interference of tablet excipients. A high value of molar absorptivity and low values of Sandell's sensitivity, LOD, and LOQ signposts the good sensitivity of proposed methods.

Assay of saxagliptin marketed dosage form
The proposed methods were applied for the assay of marketed dosage forms containing saxagliptin (label claim 5 mg). The % assay of saxagliptin found to be 100.27 and 99.86 by method-1 and method-2, respectively. There was no interference of formulation excipients during the estimation of saxagliptin in tablets. The assay values were found to be within the limits and % relative standard deviation was less than 2.

Comparison with reported analytical methods
The proposed AQbD method is found to be superior to literature methods due to its ability to predict interactive effects of parameters on performance of the method. The literature methods have limitations such as less robust, less feasibility for method transfer, no variable-interaction study and required high number of experiments. These limitations were eliminated using response surface methodology in the present investigation. Two chromogenic reagents (MBTH and ninhydrin) were employed for chemical derivatization of saxagliptin, which were not reported earlier. From Table 4, the present method was found to be highly sensitive than literature methods, owed to linearity at lower concentration range, LOD and LOQ values (0.007, 0.994). The proposed methods rely on the use of distilled water as a solvent, which point to an economical and eco-friendly method for drug analysis.

CONCLUSION
In this study, two spectrophotometric methods were developed which evaded the use of organic solvents for analysis of saxagliptin in bulk and pharmaceutical dosage form. Chemical derivatization mechanisms for saxagliptin with MBTH and ninhydrin were proposed. The optimization of reaction conditions for visible spectroscopic estimation of drug was performed through AQbD approach, where experimental design (BBD, CCD), statistical analysis and response surface analysis were employed. Proposed methods obeyed validation criterion of ICH. The assay values were in good agreement with the label claim and suggested that no interference of formulation excipients during the estimation of the drug. Contemplated methods are more sensitive, less chemical hazardous and versatile over reported methods. Hence the proposed eco-friendly and economical methods can be routinely employed in the quality control for analysis of saxagliptin in the pharmaceutical dosage forms.

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Table 1 Box-Behnken Design, Central Composite Design and experimental results

| Box-Behnken Design | Central Composite Design |
|--------------------|--------------------------|
| Run | A1 | B1 | C1 | Y1 | Run | A2 | B2 | C2 | Y2 |
| 1 | 1 | 3 | 20 | 0.584 | 1 | 1.2 | 0.2 | 15 | 0.934 |
| 2 | 0.8 | 5 | 20 | 0.577 | 2 | 1.2 | 0.6 | 5 | 0.711 |
| 3 | 1 | 3 | 20 | 0.586 | 3 | 1 | 0.4 | 10 | 0.979 |
| 4 | 1 | 5 | 10 | 0.529 | 4 | 1.28 | 0.4 | 10 | 0.948 |
| 5 | 1.2 | 3 | 10 | 0.554 | 5 | 0.72 | 0.4 | 10 | 0.725 |
| 6 | 0.8 | 3 | 10 | 0.479 | 6 | 0.8 | 0.2 | 5 | 0.688 |
| 7 | 1 | 3 | 20 | 0.582 | 7 | 1 | 0.4 | 17 | 0.967 |
| 8 | 0.8 | 3 | 30 | 0.488 | 8 | 1 | 0.4 | 10 | 0.977 |
| 9 | 1.2 | 1 | 20 | 0.486 | 9 | 0.8 | 0.6 | 15 | 0.815 |
| 10 | 1 | 5 | 30 | 0.468 | 10 | 1 | 0.4 | 10 | 0.981 |
| 11 | 1.2 | 5 | 20 | 0.571 | 11 | 1 | 0.4 | 10 | 0.845 |
| 12 | 1.2 | 3 | 30 | 0.579 | 12 | 1 | 0.68 | 10 | 0.845 |
| 13 | 1 | 3 | 20 | 0.582 | 13 | 1 | 0.4 | 10 | 0.983 |
| 14 | 0.8 | 1 | 20 | 0.405 | 14 | 1 | 0.18 | 10 | 0.789 |
| 15 | 1 | 1 | 10 | 0.455 | 15 | 1 | 0.4 | 10 | 0.982 |
| 16 | 1 | 5 | 30 | 0.567 |
| 17 | 1 | 3 | 20 | 0.581 |

A1: MBTH (%), B1: ferric chloride (%), C1: reaction time (min), Y1: absorbance of blue-green complex; A2: Ninhydrin (%), B2: NaOH (M), C2: heat time (min), Y2: absorbance of Ruhemann’s purple.

Table 2 ANOVA and regression analysis of selected models

| Response | Y1 | Y2 |
|----------|----|----|
| Fit Model | Quadratic | Quadratic |
| SD | 0.0037 | 0.0026 |
Table 3 Optimized characteristics of saxagliptin

| Parameters                              | Value                     | Method-1 | Method-2 |
|-----------------------------------------|---------------------------|----------|----------|
| Absorption Wavelength (nm)              |                           | 600      | 585      |
| Beers law range (µg/mL)                 | 0.01-0.25 µg/mL           | 1-10 µg/mL |          |
| Regression equation                     | Y = 2.7709 X + 0.307      | Y = 0.0704 X + 0.2494 |          |
| Correlation coefficient(r²)             | 0.999                     | 0.999    |          |
| Limit of Detection (µg/ml)              | 0.002                     | 0.328    |          |
| Limit of Quantification (µg/ml)         | 0.007                     | 0.994    |          |
| Molar Absorptivity (L mole⁻¹cm⁻¹)       | 1.89 X 10⁻⁴               | 0.302 X 10⁻⁴ |          |
| Sandell’s sensitivity (µg cm⁻²)          | 1.66 X 10⁻⁴               | 1.04 X 10⁻⁷ |          |
| Stability of colored species            | 7 hr                      | 5 hr     |          |

Table 4 Comparison of proposed method with literature methods of saxagliptin

| Parameter                          | Reported method | Reported method | Proposed method |
|------------------------------------|-----------------|-----------------|-----------------|
| Chromogenic reagent                | DDQ             | TCNQ            | MBTH            |
| λ_max (nm)                         | 461             | 838             | 470             |
| LOD (µg/ml)                        | 2.53            | 2.79            | 0.56            |
| LOQ (µg/ml)                        | 2.68            | 8.46            | 1.68            |
| Linearity range                    | 50-300          | 10-110          | 5-30            |
| Time of reaction (min)             | 40              | 30              | 10              |
| Reaction stability (min)           | 30 min          | 1 hr            | 4 hr            |
| Diluting solvent                   | Methanol        | Methanol        | Water           |

*DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone); TCNQ (7,7,8,8-tetracyanoquinodimethane); NQS (1,2-Naphthoquinone-4-sulfonic acid); NBD-Cl (4-chloro-7-nitrobenzofurazan); MBTH ((3-Methyl-2-benzothiazolinone hydrazone)

FIGURES
**Step 1:**

\[
\text{MBTH} \xrightarrow{\text{FeCl}_3 - 2e, H^+} \text{Electrophilic intermediate}
\]

**Step 2:**

\[
\text{Saxagliptin} \xrightarrow{\text{Electrophilic intermediate}} \text{Green coloured chromogen}
\]

**Fig. 1** Oxidative coupling reaction between saxagliptin and MBTH

**Fig. 2** Schiff's base formation and condensation of saxagliptin with ninhydrin
Fig. 3 Desirability study (2D, 3D plot)- (a): Design space for method-1; (b): Design space for method-2

Fig. 4 Effect of solvent on saxagliptin reaction with MBTH and Ninhydrin
**Fig. 5** Job’s continuous variation plot for method-1 and method-2

**Fig. 6** Overlaid UV-Visible spectra (a): saxagliptin (0.01-0.25 μg/mL) with MBTH (b): saxagliptin (0.1-10 μg/mL) with ninhydrin