Original Article

# Box-Behnken Design Assisted Optimization and Standardization of Chromatographic Methodology for Quality Assessment of Metformin: Analytical Quality by Design Avenue

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## ABSTRACT

**Background:** Metformin (MET) is an oral antidiabetic agent falls chemically under the category of biguanides and it is effectively utilized in the management of type 2 diabetes mellitus. It is marketed in the tablet dosage form and hence quality control and assessment of MET is very much essential and important. **Objectives:** The objectives of present research investigation are to implement the Box-Behnken Design (BBD) in the optimization and validation of Reverse Phase-High Performance Liquid Chromatographic (RP-HPLC) method for the quality assessment of MET and also to systematically apply the Analytical Quality by Design (AQbD) Approach. **Materials and Methods:** In methodology BBD was employed to identify and optimize the critical method aspects for augmenting the performance of proposed methodology. The optimum chromatographic elution was performed on Agilent $C_{18}$ (5$\mu$m, 250 × 4.6 mm i.d.) column employing methanol and 0.1% ortho phosphoric acid in water with pH 2.8 in the proportions of 4:96 v/v as solvent system. The elution was carried out at 0.7 mL/min flow of mobile phase and identification at 231 nm using UV detector. The BBD driven optimized method was standardized as per guidelines of International Council for Harmonisation Q2 ($R_1$) in terms of validating method parameters such as specificity, linearity, detection and quantitation limit, precision, robustness, ruggedness and accuracy. **Results:** The method was linear in the concentration of 5-25$\mu$g/ml with $R^2$ = 0.999. The detection and quantitation limit were obtained at concentration of 0.30 and 0.93 $\mu$g /ml. The values of precision, robustness, and ruggedness parameters were found to be well within the required limit of acceptance with deviation of relative standard < 2%. The accuracy of MET was observed 99.22% to 100.25% at three different recovery experiments. At the end the proposed design of experiment (DoE) oriented methodology successfully applied for quantification of MET. **Conclusion:** The BBD and AQbD approaches are highly useful for the quality assessment of MET in bulk and its marketed tablet dosage forms.

**Key words:** Analytical Quality by Design, Box–Behnken Design, Chromatography, Metformin, Standardization.

## INTRODUCTION

The quality assessment of drugs and its pharmaceutical formulations is very important and essential during product development stage in pharmaceutical industry. Analytical assessment is the critical approach in quality control of pharmaceutical products. The poor analytical assessment may lead to generation of inaccurate values, wrong data that may be risky to the drug and product formulation stage. In order to address such crucial issues and concerns, various pharmaceutical drug regulatory bodies, such as "US Food and Drug Administration" and "International Council for Harmonisation" (ICH) have been stated the quality by design (QbD) approaches and guideliens to overcome these issues. In the recent years, ICH has declared set of rules

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and guidance in ICH Q14 on development of analytical procedures and revision of Q2 (*R*1) analytical validation Q2 (*R*2)/Q14.[1]

The traditional chromatographic approach for assessment of drug molecules in bulk and dosage forms was done by a trial approach and error method. Example, by changing single parameter at single time and identify the change of the result until we get the suitable procedure was obtained. Traditional approach is very long approach or method and needed large quantity of manual result analysis. It required basically few practical runs, and in few cases, the reported procedures requires again change in method when scaled up, and in actual product development stage.[2,3]In order to overcome the limitations and disadvantages of traditional chromatographic methods we can adopt the AQbD principles as it includes the statistical DoE to know a "method operable design space" of a robust analytical procedure. The "method operable design space" gives the practically possible working areas or region in which changes to the method aspects will not reasonably affects the quality and data of the procedure. It will be technically and practically important to know if a "method operable design space" for change in chromatographic parameters can be generated to assist the optimization of a rugged and robust procedure.[4] Many scientists have implemented the AQbD approaches and guidelines to chromatographic procedures.[5-10]The systematical and rational utilization and implementation of elements of QbD to analytical procedure optimization to obtain good performance of technique is known as AQbD.[11,12] This avenue gives surety of reliability and high quality of the analytical procedures and reduces the failure risk in the phase of standardization as well as routine quality analysis. "It is a scientific and risk-based method for understanding of the critical analytical attributes (CAAs) and affected independent parameters impacting the performance of procedure". AQbD gives the detailed information on the risks and effects between the variables of the method.[13,14]MET is an oral antidiabetic agent chemically categorized to biguanides class and effectively utilized for the management of type 2 diabetes. It mainly shows its mechanism by reducing the glucose secretion. It decreases the level of cholesterol (LDL), also reported achieves the weight loss in few cases.[15] It is also used for the treatment of "polycystic ovary syndrome".[16] MET is marketed in single and also in combined dosage form with other antidiabetic drugs. In the year 1922 MET was prepared by reaction of dimethylamine hydrochloride and 2-cyanoguanine in the presence of heat.[17] The adverse effects of MET includes the lactic acidosis and few related to gastro intestinal tract. MET is contraindicated in the patients with diseases of liver, kidney, lung and heart disorders.[18] Chemically MET is N, N-dimethylimido dicarbonimidic diamide with molecular formula $C_4H_{11}N_5$ and molecular mass 129.164 gm/mol. It is soluble very freely in $H_2O$. MET is sensitive to light and degrades when heated emitting nitric oxide. It appears as white to off-white crystalline powder with pKa value 12.4. The chemical structure of MET is presented in Figure 1.

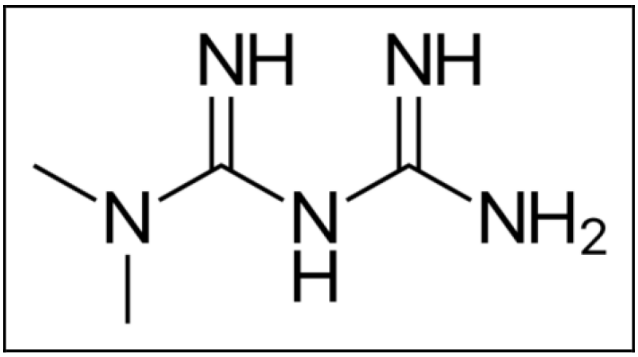


**Figure 1: Chemical Structure of MET.**

Literature review revealed that various analytical methods have been reported for quality assessment of MET in bulk and pharmaceutical dosage forms. MET is analysed by few chromatographic methods in its single and combined dosage forms. But the reported chromatographic assessment procedures suffer with various limitations and disadvantages like critical steps, complicated methods, extraction steps are multiple which leads to more time consuming procedure. Most of the published chromatographic procedures for MET have limitations as they have complex solvent system composition, longer retention time and use of costly solvents and also methods are developed with traditionally approach.19The scientific, BBD assisted and AQbD driven chromatographic optimization of MET has not discussed in detail till date. Hence there is strong and solid requirement for the optimization of simple, robust, accurate and sensitive procedure for assessment of MET employing BBD to overcome the limitations and issues as mentioned above and also to assure the quality of the proposed method.The main objectives of proposed research work is to develop and optimize an simple, sensitive, rapid, rugged, robust, accurate and reliable RP-HPLC methodology by employing BBD and AQbD approaches for quality analysis of MET in its pharmaceutical tablet dosage forms as well as in bulk powder.

## MATERIALS AND METHODS

### Reagents and Chemicals

MET (99.8%) was obtained as a gift sample from FDC Ltd. Verna-Goa. The HPLC grade methanol was purchased from Merck India Limited. The solvent system was filtered employing 0.45 µm nylon filters obtained from Pall India Private Limited, Mumbai, India. The solvent system was degassed and sonicated using sonicator. The marketed tablets of MET was obtained from local Pharmacy shops of Belagavi, Karnataka, India and analyzed for its quality using BBD assisted RP-HPLC methodology.

### Apparatus, Equipments and Chromatographic Conditions

In the proposed research work HPLC system of Agilent 1100 (Autosampler), Chemstation possessing software was used. The chromatographic separation of the MET was achieved on Agilent $C_{18}$ column (250 × 4.6 mm i.d., 5µm). The solvent system composed of methanol: 0.1% ortho phosphoric acid (OPA) in water with pH 2.8 in the proportions of 4:96 v/v. Elution of MET was done at 0.7 mL/min with UV identification at 231 nm. The temperature of column was maintained at ambient, and the injection volume of 20 µL. The Chromatographic grade water was used and it was again filtered using a 0.45 µm membrane filters (Nylon) before taking it for the preparation of solvent system composition. The micro weighing balance of Sartorius was used for weighing the standard drug and powdered sample of MET. In order to degas the solvent system as well to solubilize the drug and powdered drug sample into solvent, Sonicator of MJL lab was used. All the glassware's used such as pipettes, measuring cylinders and volumetric flasks were calibrated before use.

### Preparation of solvent system

1 mL of OPA was measured and transferred into 1000 mL milli-Q water and pH was maintained at 2.8 and filtered through 0.45-µm nylon membrane filters. The mobile phase used was methanol to 0.1% OPA in water with pH 2.8 in a ratio of 4:96 (v/v).

### Preparation of standard stock solution

The MET stock was made by dissolving 10 mg of MET in 10 ml of methanol. The resulting stock was sonicated to solubilize the MET, and then 1 ml of this solution was transferred into 10 ml of volumetric flask (VF), and it was diluted 10 ml with solvent system as diluent. This stock was utilized for optimization and validation of experimental parameters. The further working solutions of MET were obtained by serial dilutions. The series of concentrations were obtained by using solvent system as diluent. The final solutions were filtered using a 0.45 µm syringe filter. The final dilutions were then transferred to vials before chromatographic runs.

### Preparation of sample solution

Weighed and powdered 20 MET tablets. The powder amount equal to 10 mg of MET was taken and transferred into a 10 ml of the VF. Then, 2 ml of solvent system was added and 15 min sonicated to solubilize it and volume was made up to the mark with solvent system. The resulting solution, filtered using Whatman filter paper and then 1 ml of this solution diluted to 10 ml with solvent system. Further 2.5 ml of the above solution transferred in 10 ml of VF and made up to the mark with solvent system (25 µg/ml).

### Preliminary Method Development

In order to select the suitable stationary phase, initial experimental runs were performed for identification of possible combinations and selection of column and solvent system. Initial trials were carried out by using literature review. In order to develop cost effective methodology, methanol was chosen for trial study. Aqueous solvent composed of different concentration of OPA were chosen for effective separation with different pH. By the analysis of the above trials, the $C_{18}$ stationary phase was used for further study.

### Implementation of AQbD Avenue

QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management." "The approach of QbD covers a better scientific understanding of critical parameters and qualities of product, designing tests and controls based on the scientific limits of understanding during the development stage and using the knowledge and data generated during the life cycle of the product to work on a constant improvement". In the present investigation we have worked on various elements of AQbD and implemented in the practical methodology. It has started with identification of Analytical Target Profile (ATP), followed by study of Critical Quality Attributes (CQAs) and Risk Assessment (RA) by employing fish-bone approach. Further approach is mainly deals with application of DOE which helps in the factor screening and optimization of RP-HPLC methodology.

### Analytical Target Profile (ATP)

Identification and setting up the ATP is first and most important step in the QbD based analytical procedure

development and it can be defined as collection of information required for desired quality profile needed for the development of method. This helps for assuring the safety, quality, and practical usefulness of the analytical procedure at the site of commercial application of it in the pharmaceutical industry. Various components of ATP include the "target drug, target samples and method application, analytical method, instrument characteristics and requirements, sample preparation steps, and analytical procedure quality attributes".

### Critical Quality Attributes (CQAs)

In order to meet the requirements of target profile of analytical procedure, various CQAs need to be selected and it is very important and essential step in the AQbD based method development. The CQAs can be defined as "the measurable parameters of the chromatogram of analyte that should be within an appropriate and acceptance limit or range or criterions to warrant the desired quality and ability of the analytical procedure". In case of chromatographic development usually the critical quality attributes includes the tailing factor (TF), retention time (RT), peak area (PA), peak spectral purity, theoretical plates (TP), and assay limit etc. Out of these attributes, RT, TP, and TF have real impact on the performance of analytical procedure.

### Risk Assessment (RA)

The assessment of risk in the very important and essential step in the QbD based procedure development. In this study RA was conducted for determining the probability of failures in the procedure. In order to perform this, a cause to effect model for relationship of "critical material attributes" and "critical method parameters" with the Critical Analytical Attributes (CAAs) was done and executed by construction of an "Ishikawa Fish-Bone" (IFB) diagram.

### Factor Screening by Design of Experiment Avenue using Box-Behnken Design (BBD)

A new chromatographic procedure was optimized for MET using software of Design Expert. In this software, design of Box-Behnken was employed to optimize the "Critical Process Parameters" (CPPs) or "Critical Method Parameters" (CMPs), and to assess the interaction of these parameters on the CQAs. BBD is a three factor two level design and it requires fewer experimental runs.

### Box-Behnken Optimization Study Design

The chromatographic optimization of parameters was done by utilizing 3 factors, 2 levels BBD to estimate the main, effects and quadratic effects of critical factors on the variables of identified responses.[20-22] The BBD composed of 14 experiment runs and screening phase includes the following steps. In the first phase selection of CMPs was done which includes the flow rate, organic phase (% methanol), and wavelength of detection. The second phase of study includes selection of CQAs. In the present study CQAs selected are RT, PA, TP, and TF. These responses were monitored during the practical trials.

### Method Development According to Experimental Design

As per factor screening method, the selection of the CMPs which are affecting the performance of procedure was optimized employing 3 factor at two equidistant levels (low (-1) and high (+1) levels). A 10µg/mL of MET was used for all chromatographic runs, and analyzed for CAAs (RT, PA, TP, and TF).

### Data Analysis and Model Validation

The responses generated after running suggested trial were added to software and various plots like "3D response surface plots" and "graph plots" were obtained. These plots suggest the effect of CMPs on the selected CQAs. The assessment of these plots was utilized to analyse as to which parameter give the reasonable responses. Based on these data, the final CMPs of the procedure were identified and the optimized conditions were selected. The statistical tool, like "analysis of variance" (ANOVA) for every response was used to study the significance of each parameter used for the study using the $p$ value.

### Method Validation

In order to standardize the suitability of the proposed method for its intended use, method validation was carried out as per ICH guidelines by assessing the system suitability, linearity and range, detection and quantification limit, precision, ruggedness, robustness and accuracy. The proposed RP-HPLC procedure was then applied successfully for the quality assessment of MET in its marketed table forms.

### System Suitability

The suitability of system and testing is an essential part of any analytical method. It was performed by injecting three replicates of 10 µg/mL standard MET solution and then evaluating the each chromatogram with respect to its RT, TP and TF.

### Specificity and Selectivity

The specificity and selectivity of the procedure was done by obtaining the HPLC peak of solution of MET (10 µg/mL), sample and blank chromatogram of solvent system. It helps to identify the analyte, interference from other peaks, and also the peak purity.

### Linearity and Range

The linearity of procedure was assessed between the ranges of 5 to 25 µg/mL of MET. The linearity response was determined by preparing different concentrations and injecting three times into HPLC, and recording chromatograms and noting down the peak area for all the graphs. The standard calibration curve was graphed as peak area *vs* the concentration of the MET in µg/mL.

### Detection and Quantification Limit

"Limit of detection (LOD) is the lowest amount of drug in a sample that can be detected but not necessarily quantified as an exact value". Detection limit was obtained using the following equation:

$$LOD = 3 \times \sigma/S$$

Limit of quantification (LOQ) of the developed method is "the lowest amount of analyte or drug in a sample which can be quantified with suitable precision and accuracy". Quantification limit was obtained using the following equation:

$$LOQ = 10 \times \sigma/S$$

"Where $\sigma$ is the standard deviation of $y$-intercepts of regression lines, and $S$ is the slope of the calibration curve" of MET.

### Precision

The precision is estimated in terms of relative standard deviation **(**RSD). The precision was tested by injecting three replicate injections of concentration 10, 15 and 20µg/mL. The intraday and interday precision study was done by injecting 3 replicates of different concentration of MET and calculating %RSD for PA. (It was assessed on the same day and on three different days).

### Robustness

The robustness of the method was evaluated by injecting the standard solution of MET at deliberately changing the chromatographic conditions which includes a change in composition of organic phase in solvent system, flow rate, and wavelength.

### Ruggedness

The ruggedness of the proposed method has been done by analyzing the standard solution of MET by two different analysts. The overall mean, and % RSD was calculated for peak area obtained in the chromatogram of each peak.

### Accuracy

Accuracy of the method was assessed from the recovery study of MET through 5µg/ml solution of MET spiked with 80, 100, and 120%. The mean recovery and % RSD was calculated at each level.

### Quality Assessment of MET using Box-Behnken Design Assisted RP-HPLC Method

Weighed and powdered 20 MET tablets. The powder amount equal to 10 mg of MET was taken and transferred into a 10 ml of the VF. Then, 2 ml of solvent system was added and 15 min sonicated to solubilize it and volume was made up to the mark with solvent system. The resulting solution, filtered using Whatman filter paper and then 1 ml of this solution diluted to 10 ml with solvent system. Further 2.5 ml of the above solution transferred in 10 ml of VF and made up to the mark with solvent system (25 µg/ml) and injected into stabilized HPLC for further elution and analysis.

## RESULTS AND DISCUSSION

### Initial Method Development

In order to optimize a simple, cost-effective, precise and accurate method for assessment and quality control of the MET in its bulk as well as pharmaceutical tablet dosage forms, preliminary experiments on the basis of literature search and trial and error basis were carried out. Initially, development was started with selecting mobile phase by utilizing various combinations of solvents such as methanol, and o-phosphoric acid. The buffer phase is tried at different concentrations in order to achieve better separation of the MET. In initial runs, it was ientified that the selection of OPA in 0.1% concentration with pH 2.8 which leads to faster and effective separation of drug with elution of MET at lower RT along with low peak tailing and satisfactory peak symmetry.

### Analytical Quality by Design Approach and its Implementation

Various approaches and methodologies of AQbD were applied to proposed RP-HPLC method as discussed in methods section. The ATP, CQAs and process parameters of method were studied, identified and presented in Table 1.

## Risk Assessment and Screening Studies

RA study was performed to analyze the CMPs, which are high-risk factors and have a critical effect on the CAAs. In this study, IFB diagram (Figure 2) was prepared to list out serious risk factors that may have an effect on performance of method. The risk factors listed may includes the method of extraction of sample, time of extraction, solvent used for extraction etc. and instrumental related parameters such as ratio of solvent system, chromatographic mode, flow rate, and injection volume. The high-risk procedure variables were listed and exposed to further analysis by applying suitable experimental optimization design. The high-risk method variables selected as flow rate of mobile phase, % composition of organic phase (methanol) in mobile phase and wavelength of detection.

**Table 1: QTPP, CQAs and Process Parameters of Proposed RP-HPLC Method.**

| Sl. No. | AQbD Parameters | Description |
|---|---|---|
| 1 | Quality target product profile | Flow rate, mobile phase composition, wavelength of detection, pH, temperature, and column attributes, injection volume, pressure, buffer attributes. |
| 2 | Critical quality attributes | Retention time, Peak area, theoretical plates, peak purity, tailing factor. |
| 3 | Process parameters | Specificity, selectivity, linearity, range, precision, robustness, ruggedness, accuracy, LOD, LOQ. |

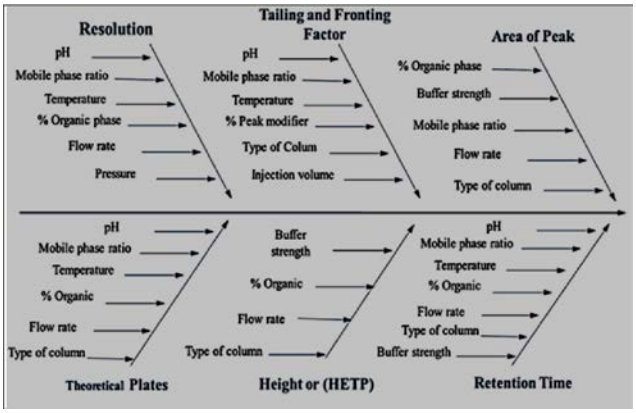


**Figure 2: Ishikawa Fishbone Diagram.**

## Box–Behnken Optimization Design

It is very important component of DOE avenue and in order to implement for the RP-HPLC method, we have selected high-risk factors such as flow rate of mobile phase, % organic phase in mobile phase and wavelength of detection. They were optimized by BBD to detect main, interaction and quadratic effects of these factors on RT, PA, TP and TF. The 14 runs were performed, and obtained results were assessed statistically using software. The independent variables selected were flow rate of mobile phase ($X1$), % organic phase in mobile phase, and wavelength of detection, whereas RT, PA, TP, and TF were selected as the dependent responses. The BBD optimization is shown in Table 2. The software carried analysis of variance (ANOVA), and statistical optimization. The ANOVA results are presented in Table 3. This implies that the model is valid. The "3D-surface" and "2D-contour plots" were also analyzed to define design space and to visualize the effect of independent parameters and their effects on the

**Table 2: Box–Behnken Optimization Design.**

| | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 | Response 3 | Response 4 |
|---|---|---|---|---|---|---|---|
| Run | A:Flow rate | B:Methanol | C:Wavwlength | RT | PA | TP | TF |
| | ml/min | % | nm | | | | |
| 1 | 0.7 | 3 | 230 | 3.45 | 4850.37 | 11675 | 0.82 |
| 2 | 0.8 | 2.3 | 231 | 2.95 | 4019.71 | 10892 | 0.84 |
| 3 | 0.7 | 3 | 232 | 3.369 | 4521.28 | 11693 | 0.83 |
| 4 | 0.6 | 4 | 231 | 3.89 | 5516.24 | 12789 | 0.8 |
| 5 | 0.7 | 5 | 230 | 3.33 | 4896.5 | 11458 | 0.83 |
| 6 | 0.9 | 3 | 232 | 2.61 | 3555.04 | 9810 | 0.86 |
| 7 | 0.7 | 5 | 232 | 3.32 | 4665.06 | 11373 | 0.82 |
| 8 | 0.8 | 4 | 229.3 | 2.92 | 4373.36 | 10645 | 0.84 |
| 9 | 0.9 | 3 | 230 | 2.62 | 3755.37 | 9777 | 0.85 |
| 10 | 0.8 | 4 | 231 | 2.62 | 3707.75 | 9733 | 0.86 |
| 11 | 0.9 | 5 | 230 | 2.71 | 4018.88 | 9950 | 0.86 |
| 12 | 0.9 | 5 | 232 | 2.69 | 3785.56 | 9793 | 0.85 |
| 13 | 0.8 | 5.7 | 231 | 3 | 4326.7 | 10679 | 0.84 |
| 14 | 0.8 | 4 | 232.7 | 3 | 4484.22 | 10716 | 0.84 |

**Table 3: Statistical Analysis Data.**

| ANOVA Parameters | Retention Time | Peak Area | Theoretical Plates | Tailing Factor |
|---|---|---|---|---|
| *R*-Squared | 0.9960 | 0.8277 | 0.9970 | 0.9987 |
| Adjusted *R*-squared | 0.9913 | 0.8133 | 0.9936 | 0.9971 |
| Predicted *R*-Squared | 0.9583 | 0.7698 | 0.8559 | 0.8529 |
| Standard Deviation | 0.0363 | 239.06 | 74.53 | 0.0009 |
| %C.V | 1.19 | 5.53 | 0.69 | 0.1116 |
| *F*-value | 211.69 | 57.63 | 288.01 | 646.40 |
| *p*-value | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

*R*-squared = Coefficient of determination.
*F*-value = Value on the F distribution.
*p*-value = Probability of falsely detecting a significant effect.
C.V.% = Percent Coefficients of variance.

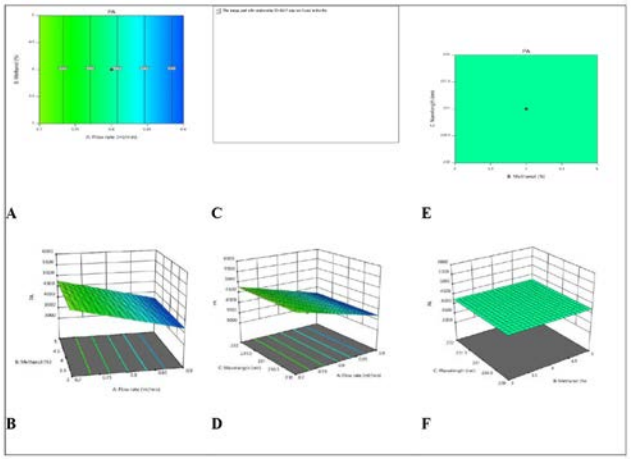


**Figure 4: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on peak area, effect of flow rate and wavelength (C and D) on peak area, %methanol and wavelength (E and F) on peak area.**

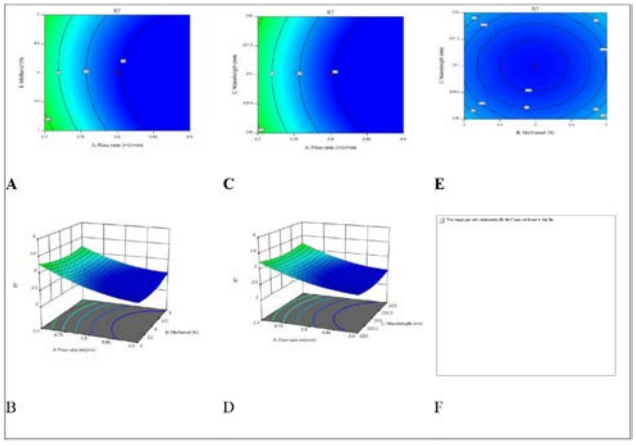


**Figure 3: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on retention time, effect of flow rate and wavelength (C and D) on retention time, %methanol and wavelength (E and F) on retention time.**

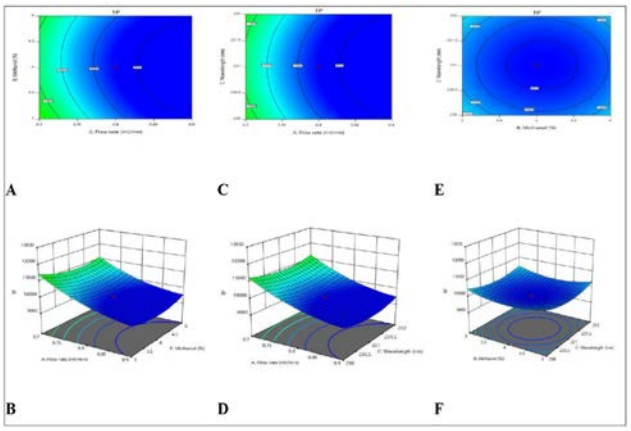


**Figure 5: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on theoretical plates, effect of flow rate and wavelength (C and D) on theoretical plates, %methanol and wavelength (E and F) on theoretical plates.**

respective response variables. As the proposed model has more than two independent factors, one factor was kept constant for each plot. Figure 3-6 presents the portrays 3D surface plot, and it's corresponding 2D contour plot showing the effects of flow rate, % composition of organic phase, and wavelength on RT, PA, TP and TF.

## Analytical method validation

In the system testing, parameters, like RT, TP, and TF were assessed and %RSD was calculated prior to perform the analysis. Figure 7A presented the HPLC chromatogram of standard MET with RT 2.96 min. Figure 7B shows the chromatogram of MET in sample with $R_t$ 2.96 min. The blank chromatogram also showed no appearance of any peak at $R_t$ of

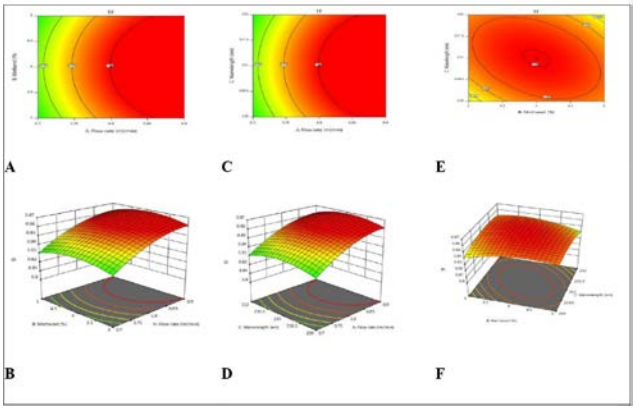


**Figure 6: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on tailing factor, effect of flow rate and wavelength (C and D) on tailing factor, %methanol and wavelength (E and F) on tailing factor.**

MET. No interference observed at the retention time of MET peak in standard and sample chromatogram; thus, method was found to be specific and selective for MET. The overlay chromatograms of MET were presented in Figure 7C. The standard calibration curve of MET was plotted in the concentration range of 5 to 25 µg/mL (Figure 8). The procedure was linear in the concentration range of 5-25 µg/mL with $R^2$ value 0.999. Limit of detection and limit of quantitation were found to be 0.30 and 0.93 µg /ml respectively. Data in Table 4 suggest the precision, robustness, and ruggedness of proposed method. The %RSD of each parameter for standardization falls within the limit (<2%). The robustness of procedure was assessed by changing solvent composition methanol: aqueous phase (3:97 to 5:95 v/v), change in wavelength from 230 to 232 and change in the flow rate from 0.6 to 0.8 ml/min. Acceptable %RSD values were obtained after making small deliberate variations in developed method parameters. This suggests that the procedure is robust for the industrial analysis. The ruggedness of the procedure was done by different analysts and values of results were found to be within the acceptance. An recovery with %RSD of less than 2% was obtained from all 3 level recovery studies. The %RSD of all the 3 levels is presented in Table 5.Quality Assessment of MET by Box–Behnken Optimized RP-HPLC Method

The BBD driven RP-HPLC analysis of marketed formulation of MET presented excellent recovery. The % recovery was found to be 99.89%. The retention time of MET in dosage was not changed with regard

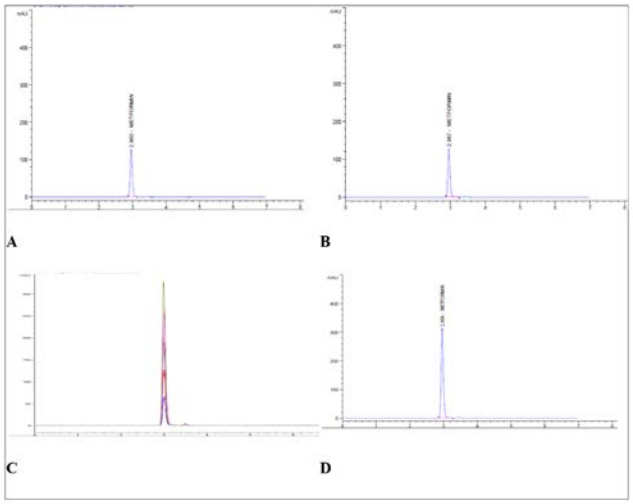


**Figure 7: Chromatograms depicting (A) MET Standard (B) MET sample (C) Overlay of MET Standard (D) MET assay sample.**

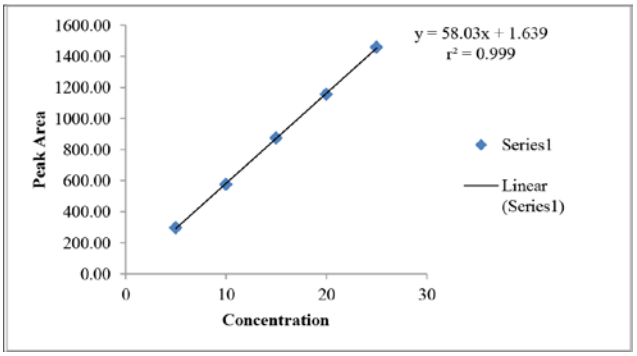


**Figure 8: Standard Calibration Curve of Metformin.**

**Table 5: Accuracy Study Data of MET.**

| Level | Sample | Recovery (%) | Mean Recovery (%) | % RSD |
|---|---|---|---|---|
| 80% | S1 | 99.18 | 98.98 | 0.69 |
| | S2 | 99.53 | | |
| | S3 | 98.22 | | |
| 100% | S1 | 99.84 | 99.79 | 0.48 |
| | S2 | 99.29 | | |
| | S3 | 100.25 | | |
| 120% | S1 | 99.79 | 99.20 | 0.70 |
| | S2 | 99.38 | | |
| | S3 | 98.44 | | |

**Table 4: Analytical Method Validation Report.**

| Precision | Intraday | | Interday-1 | | Interday-2 | | Interday-3 | |
|---|---|---|---|---|---|---|---|---|
| Conc. | Peak Area | %RSD | Peak Area | %RSD | Peak Area | %RSD | Peak Area | %RSD |
| 10 µg/mL | 579 | 0.12 | 585 | 0.29 | 577 | 0.65 | 581 | 0.71 |
| 15 µg/mL | 873 | 0.37 | 878 | 0.31 | 876 | 0.11 | 876 | 0.13 |
| 20 µg/mL | 1163 | 0.15 | 1175 | 0.29 | 1176 | 0.80 | 1159 | 0.24 |
| Ruggedness by Change in the Analyst | | | Robustness | | | | | |
| | | | Flow Rate | | Mobile Phase | | Wavelength | |
| 5 µg/mL | 297 | 0.39 | 25 µg/mL | | 25 µg/mL | | 25 µg/mL | |
| 15 µg/mL | 876 | 0.12 | 1637 | | 1443 | | 1485 | |
| 25 µg/mL | 1457 | 0.21 | 0.19 | | 0.84 | | 0.11 | |

to MET standard. The TF and TP factor were found to be within the limits of acceptance. All the solutions of samples showed no additional peaks in chromatograms that showed no inference of dosage form additives with MET. This showed ability of proposed RP-HPLC method for routine quality assessment and analysis of MET in its bulk and tablet forms. The assay chromatogram is presented in Figure 7D.

## CONCLUSION

The present investigation successfully implemented the BBD and AQbD avenue to optimize the RP-HPLC procedure for MET assessment with a proper understanding of the critical factor response relationship for augmenting the procedure performance. The BBD and AQbD assisted RP-HPLC method optimization of MET ensured the robustness of the analytical procedure before standardization studies. This novel pathway helps the researcher and analyst to set control strategies to reduce the unwanted effect of these critical method variables on performance of method. The validation reports confirmed the specificity, selectivity, excellent linearity, detection and quantification limit, accuracy, precision robustness, and ruggedness of proposed method. The BBD assisted optimized and validated RP-HPLC method further utilized for the quality assessment of marketed MET tablets to ratify the applicability of the proposed procedure.

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## ABBREVIATIONS

**AQbD:** Analytical Quality by Design; **ANOVA:** Analysis of Variance; **ATP:** Analytical Target Profile; **BBD:** Box-Behnken Design; **CPPs:** Critical Process Parameters; **CMPs:** Critical Method Parameters; **CAAs:** Critical Analytical Attributes; **CQAs:** Critical Quality Attributes; **DOE:** Design of Experiment; **IFD:** Ishikawa Fish-Bone Design; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **MET:** Metformin; **OPA:** Ortho Phosphoric Acid; **PA:** Peak Area; **QbD:** Quality by Design; **RA:** Risk Assessment; **RSD:** Residual Standard Deviation; **RT:** Retention Time; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **TF:** Tailing Factor; **TP:** Theoretical Plates.

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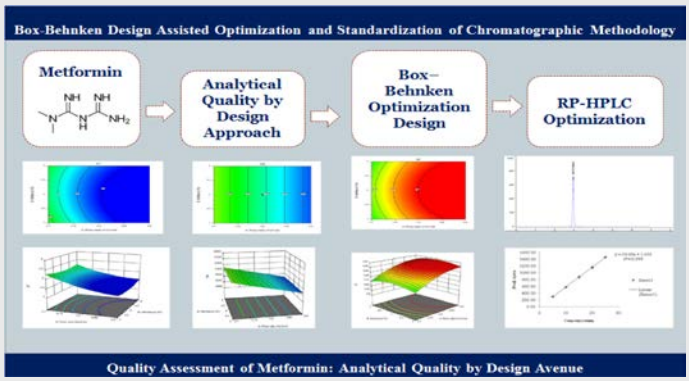
## SUMMARY

In methodology BBD was used to identify and optimize the critical process parameters for augmenting the performance of proposed methodology. The optimum RP-HPLC separation was achieved on Agilent $C_{18}$ column (250 x 4.6 mm i.d., 5µm) employing methanol and 0.1% ortho phosphoric acid in water with pH 2.8 in the proportions of 4:96 v/v as solvent system. The elution was carried out at the flow rate of 0.7 mL/min and detection at 231 nm using UV detector. The BBD driven optimized method was standardized as per guidelines of International Council for Harmonisation Q2 ($R_1$) in terms of validating method parameters such as specificity, linearity, Limit of detection and limit of quantitation, precision, robustness, ruggedness and accuracy. The method was found to be linear in the concentration range of 5-25µg/ml with $r^2 = 0.999$. The detection and quantitation limit were obtained at concentration of 0.30 and 0.93 µg /ml. The values of precision, robustness, and ruggedness parameters were found to be well within the acceptance limits with relative standard deviation < 2%. The accuracy of MET was observed 99.22% to 100.25% at three different recovery experiments. At the end the proposed design of experiment (DOE) oriented methodology successfully applied for quantification of MET. In conclusion, the BBD and AQbD approaches are highly useful for the quality assessment of MET in bulk and its marketed tablet dosage forms.

## PICTORIAL ABSTRACT



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