Ind. J. Pharm. Edu. Res., 2025; 59(2s):s689-s704.
https://www.ijper.org

Original Article

Quality by Design (QbD) Based Development and Validation of RP-HPLC Method for Simultaneous Estimation of Metformin and Teneligliptin in Bulk and their Pharmaceutical Formulation

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ABSTRACT

Aim: To develop and validate simple, accurate, precise, sensitive and robust Reversed Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous estimation of metformin and teneligliptin in bulk and their pharmaceutical formulation by using Quality by Design (QbD) approach. **Materials and Methods:** The factor screening studies were performed using 2 level 4 factor (16 run) full factorial design. C_{18} Gracesmart column (150×4.6 mm, 5 µ) was saturated with mobile phase acetate buffer pH 4: acetonitrile (56.4: 43.6 v/v). Mobile phase was pumped at flow rate 1 mL/min. Both the drugs metformin and teneligliptin detected by diode array detector at wavelength 250 nm. **Results:** Retention time for metformin and teneligliptin were found to be 1.983 min and 3.263 min respectively. Metformin and teneligliptin show linear response in concentration range 50-300 µg/mL and 2-12 µg/mL respectively. % Recovery for metformin and teneligliptin was found to be 100.15%-101.43% and 98.76%-99.48% respectively. In precision study % relative standard deviation was found less than 2 for metformin and teneligliptin. Limit of Detection (LOD) for metformin and teneligliptin was found to be 1.281 µg/mL and 0.464 µg/mL respectively. Limit of Quantification (LOQ) for metformin and teneligliptin was found to be 3.881 µg/mL and 1.407 µg/mL respectively. **Conclusion:** The proposed method was found to be accurate, precise, sensitive and robust. As a result, it might be successfully employed for the routine analysis of metformin and teneligliptin in bulk and their pharmaceutical dosage forms.

Keywords: Metformin, Teneligliptin, RP-HPLC, Quality by design (QbD) approach.

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Received: 18-03-2023;
Revised: 30-07-2024;
Accepted: 20-12-2024.

INTRODUCTION

Quality by Design (QbD) is defined as "A systematic approach to method development based on sound science and quality risk management that begins with predefined objectives and emphasizes product and process understanding and process control".[1] Analytical science plays a crucial role in the development of pharmaceutical products, as it is integrated throughout the entire product life cycle. Analytical QbD is a science-based and risk-focused approach to developing analytical methods. Its goal is to control the key method variables and improve method performance, robustness, ruggedness and flexibility. The main aim of analytical QbD is to anticipate potential failure modes, establish a robust design region and maintain the method through continuous life cycle management.[2]

Type 2 diabetes mellitus is a persistent health issue characterized by elevated levels of glucose in the blood. The condition arises when the body fails to produce sufficient insulin or if produced, it doesn't work effectively in the body. To manage this, a combination of Metformin (MET) and Teneligliptin (TEN) is commonly used. MET and TEN belongs to the group of medications called 'antidiabetic agents' used in type 2 diabetes mellitus.[3,4] Chemically MET is 1-carbamimidamido-N,N-dimethylmethanimidamide (Figure 1). It belongs to the biguanide class of antidiabetic drug. It activates liver enzyme Adenosine Monophosphate Activated Protein Kinase (AMPK) that play important role in insulin signaling, whole body energy balance and metabolism of glucose and fats.[4] TEN is chemically{(*2S*,*4S*)-4-[4-(3-methyl-1-phenyl-1H-pyrazol-5-yl)-1-piperazinyl]-2pyrrolidinyl}(1,3-thiazolidin-3-yl)methanone (Figure 2). It is class of oral hypoglycemic drug that block Dipeptidyl Peptidase-4 (DPP-4)

DOI: 10.5530/ijper.20253717

enzyme, this enzyme is responsible for degradation of Glucagon like Peptide-1 (GLP-1). TEN exerts its activity through elevation of activated GLP-1 levels which inhibit glucagon release, which in turn increases insulin level in body and decreases blood glucose levels.[4,5]

The literature survey reveals that various simple analytical methods are available like UV spectrophotometric methods,[6-10] High-performance thin layer chromatography (HPTLC) methods,[11-13] High Performance Liquid Chromatography (HPLC) methods[14-37] and LC-MS/MS Method[38] for estimation of MET and TEN individually or in combination with another drug. The primary goal of the present research is to develop and validate RP-HPLC method for simultaneous estimation of MET and TEN in bulk and their pharmaceutical formulation using a QbD approach.

MATERIALS AND METHODS

Instrumentation

The HPLC system was of thermo scientific (ultimate 3000 model) which consisted of C_{18} Gracesmart column (150 x 4.6 mm, 5 µ), P2230 plus HPLC pump and diode array detector. The auto sampler injection system used was a 20 µL sample loop. For weighing of standards and samples analytical balance (AUX-220, Shimadzu) was used. Ultrasonicator (Toshcon, Toshniwal) was used for sonication of mobile phase, standard and sample preparation.

Reagents and Chemicals

Ammonium acetate, triethylamine, acetonitrile, glacial acetic acid and HPLC grade water were procured from S.D. Fine Chem Ltd, Mumbai. MET and TEN were provided as a gratis sample by Zydus Healthcare Ltd, Sikkim. Tenglyn M (500 mg MET and 20 mg TEN) tablet was purchase from local market.

Chromatographic conditions

Gracesmart C_{18} column equilibrated with mobile phase comprising of acetate buffer (pH 4.0): acetonitrile (56.4: 43.6 v/v). Mobile phase flow rate was maintained 1 mL/min and eluents were monitored at 250 nm by using diode array detector. A 20 µL sample was injected using a fixed loop and all the chromatographic separations were carried out at 30°C temperature.

Preparation of mobile phase

Accurately weighed 3.9 gm of ammonium acetate and transferred into 1 L volumetric flask containing 200 mL water. Add 1 mL triethylamine in resulting solution and then diluted to 1 L with water. pH 4 adjusted with glacial acetic acid. The solution was then filtered through membrane filter (0.45 µ). The filtrate sonicated for 15 min. The mobile phase was prepared by mixing the prepared buffer solution with acetonitrile in the ratio 56.4: 43.6 v/v. The mixture was then sonicated for 15 min.

Preparation of stock solution of MET and TEN

50 mg of MET and 2 mg of TEN were weighed and transferred into a 100 mL volumetric flask. Add 40 mL mobile phase and sonicate for 10 min. The flask was then filled to the mark with the mobile phase to produce concentration of 500 µg/mL of MET and 20 µg/mL of TEN.

Selection of Wavelength

MET (10 µg/mL) and TEN (10 µg/mL) solutions were made in mobile phase and scanned individually in UV wavelength region 200-400 nm. MET and TEN both showed significant absorbance at 250 nm. Hence 250 nm wavelength was selected for the study.

QbD and Design of Experiment (DoE) approach

To improve chromatographic results, the impact of various chromatographic variables must be taken into consideration. These variables include mobile phase pH (A), solvent ratio (B), temperature (C) and flow rate (D) were studied. A 2^4 full factorial design was applied (Table 1) and the responses were utilized to create the design space. The resulting chromatograms were recorded. Resolution (Rs), Capacity Factor (CF), Peak Asymmetry (PA) and Theoretical Plates (TP) of drugs peaks were determined and were used to evaluate the effects of factors and factors interactions. The design along with the experimental runs and coded factors is shown in Table 2. The responses (Resolution, Capacity Factor, Peak Asymmetry and Theoretical Plates of MET and TEN) obtained is shown in Table 3.

Method validation

According to the International Conference on Harmonization (ICH) Q2R1 guideline, the proposed method was validated.

Linearity

Aliquots 1, 2, 3, 4, 5 and 6 mL of standard stock solution were pipette out and transfer in to separate 10 mL volumetric flasks. Make up the volume up to the mark with the mobile phase to give MET concentration range of 50-300 µg/mL and TEN concentration range of 2-12 µg/mL. A calibration curve was constructed by plotting the peak area versus the concentration and calculating the regression equation.

Accuracy (Recovery study)

Accuracy was established by determining the % recovery of MET and TEN through the standard addition procedure. A known quantity of standard solutions of MET and TEN were introduced into pre-determined sample solutions of MET (100 µg/mL) and (TEN 4 µg/mL) at varying levels of 50%, 100% and 150%. The percentage of recovered sample was calculated which provides a measure of the accuracy of the method.

Intermediate precision

The precision of the proposed method was evaluated both intra-day and inter-day by analyzing the responses at three different concentrations of MET (50, 100 and 150 µg/mL) and TEN (2, 4 and 6 µg/mL) three times on the same day and over three consecutive days. The relative standard Deviation (RSD) was calculated and used to report the precision results. This was done to ensure that the proposed method is reliable and consistent in measuring the concentrations within the calibration range.

Repeatability

The response of 6 solutions of MET (100 µg/mL) and TEN (4 µg/mL) was repeatedly measured. No alterations were made to the parameters of the method during this process. The results were recorded as relative standard deviation values.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated using the standard deviation of intercepts and slope of the calibration curves of MET and TEN. The LOD and LOQ were determined using the following mathematical formulae:

$$LOD = 3.3 \times \frac{\sigma}{s}$$

$$LOQ = 10 \times \frac{\sigma}{s}$$

Where, σ=Standard deviation of the response,

S=Slope of the calibration curve.

Robustness

The robustness of the method was evaluated through an investigation of the impact of variations in method parameters on the sample results of MET and TEN. The flow rate was deliberately altered by ±0.2 mL/min and the corresponding changes in the response of MET and TEN were observed and recorded. The robustness of the method was determined by comparing the altered results with the original ones.

Assay of tablet dosage form

20 Tenglyn M tablets were accurately weighed and mechanically pulverized to produce a powder equivalent to 50 mg of MET and 2 mg of TEN. The resulting powder was transferred to a 100 mL volumetric flask and 60 mL of the mobile phase was added. The mixture was then subjected to sonication for 10 min to facilitate complete dissolution of the drugs. The volume of the solution was then adjusted to 100 mL with the addition of the mobile phase. 2 mL aliquot of the solution was then diluted with 10 mL of the mobile phase to obtain a final concentration of 100 µg/mL for MET and 4 µg/mL for TEN.

RESULTS

Response 1: Resolution

The results of the statistical analysis in Table 4 (a) reveal that the Model F-Value of 181.79 is statistically significant, with a probability of 0.01% of being due to noise. This implies that the model terms A, B and AB are deemed significant as indicated by the "Prob>F" values, which are less than 0.0500. Table 5 (response 1) further supports the validity of the model by displaying a high degree of agreement between the "Pred R-Squared" and "Adj R-Squared" values of 0.9721 and 0.9837 respectively. Additionally, the "Adeq Precision" value of 31.365, which represents the signal to noise ratio, demonstrates an adequate signal as the ratio is greater than 4. Overall, these results indicate that the model can be effectively utilized for navigating the design space. Figure 3 shows the Pareto chart for effect of factor and factor interactions on resolution of MET and TEN. It indicated an increase in factors A and D and factor interaction AB decreased resolution of MET and TEN. Positive coefficients of factors B and C indicated its positive effect on resolution of MET and TEN. Negative coefficient of A and D factor and AB factor interaction indicated its negative effect on resolution of MET and TEN. Only the factors A and B and interaction AB were with significantly higher t-value than Bonferroni limit. Figure 4 shows 3D response surface plot for effect of A and B on resolution of MET and TEN. The higher coefficients of factor B compared to A indicate its greater effect on the resolution of MET and TEN.

Response 2: MET PA

Table 4 (b) presents the results of the statistical analysis of the model with the Model F-value being 83.52. The significance of the model is indicated by the low probability (0.01%) of obtaining this large value due to random noise. The results of the analysis of the individual model terms, as indicated by the "Prob>F" values,

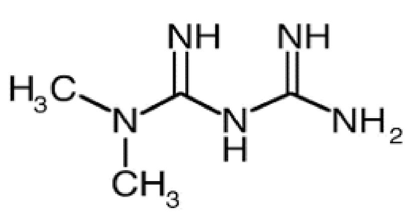

Figure 1: Chemical structure of MET.

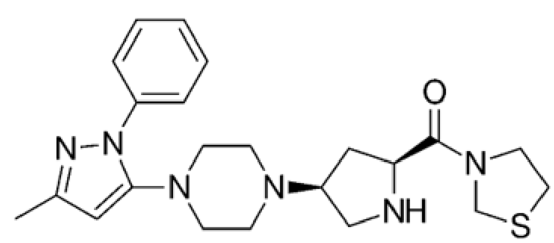

Figure 2: Chemical structure of TEN.

demonstrate that terms A, B, C, D, AB and BC are significant. The prediction accuracy of the model is evaluated in Table 5 (response 2), with the "Pred R-Squared" and "Adj R-Squared" values of 0.9442 and 0.9706 respectively. These values indicate a strong correlation between the observed and predicted responses, supporting the validity of the model. Additionally, the "Adeq Precision" measure, with a ratio of 28.723 suggests an adequate signal-to-noise ratio, making the model suitable for navigation in the design space. Figure 5 shows the Pareto chart for effect of factor and factor interactions on peak asymmetry of MET. It indicated an increase in factors B and C and interaction BC increased PA of MET. Positive coefficients of factor B and C and interaction BC indicated its positive effect on PA of MET. It indicated an increase in A and D factors and AB factor interaction decreased PA of MET. Negative coefficients of A and D factors and AB factor interaction indicated its negative effect on PA of MET. Only the factors A, B and C and interaction AB were with significantly higher t-value than Bonferroni limit. Figure 6 shows 3D response surface plot for the effect of A and B on PA of MET, it indicates decrease in A decrease PA of MET and increase in

Table 1: 2 level 4 factorial design.

Level	Factor			
	pH	Composition of Mobile phase (%v/v)	Flow rate mL/min	Temperature (°C)
-1	4	40:60	0.8	30
1	5	60:40	1.0	50

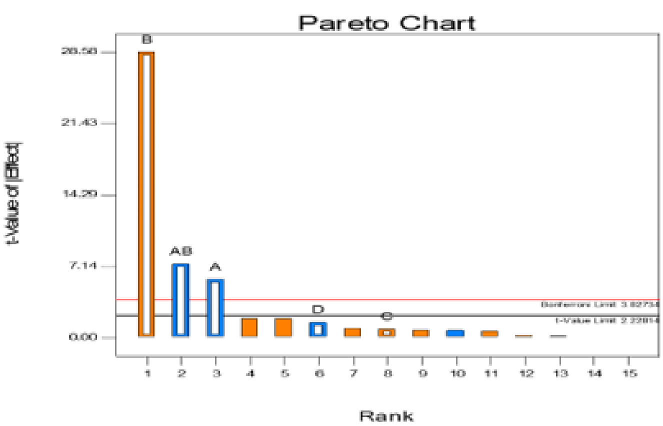

Figure 3: Pareto chart showing effects of factors and factors interations on resolution of MET and TEN.

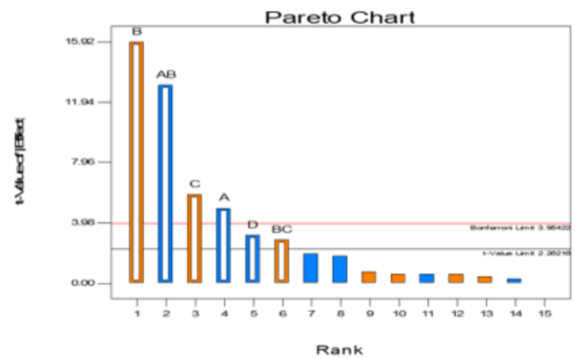

Figure 5: Pareto chart showing effects of factors and factors interactions on peak asymmetry of MET.

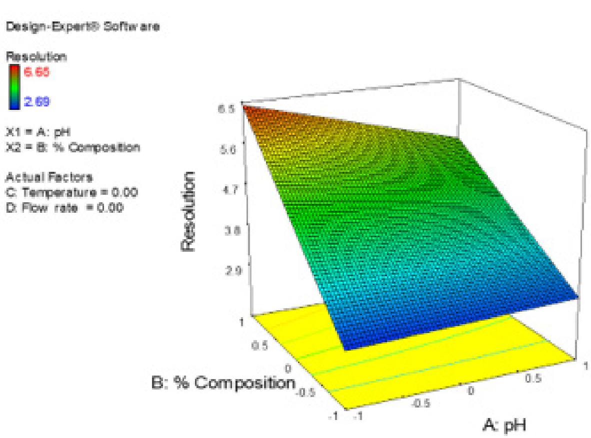

Figure 4: 3D response surface plot showing effect of % Composition and pH on resolution of MET and TEN.

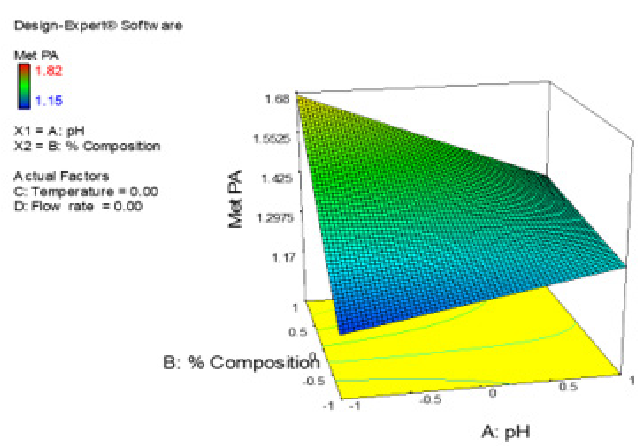

Figure 6: 3D response surface plot showing effect of A and B on Peak asymmetry of MET.

B increased PA of MET. The effect of A and B was non-linear. Figure 7 shows 3D response surface plot for the effect of B and C on PA of MET. It indicates an increase in PA of MET. The effect of B and C was linear with positive coefficient.

Response 3: TEN PA

The results of the analysis, presented in Table 4 (c), demonstrate that the model is statistically significant as evidenced by a low probability of occurrence (0.02%) of the obtained "Model F-Value" (16.33) due to random noise. The "Prob>F" values, which assess the significance of individual model terms, indicate that the terms B, C, AB and AC are statistically significant (Prob>F<0.0500). On the other hand, model terms with values greater than 0.1000 are considered not significant, which suggests the possibility of improving the model by reducing the number of these terms. The "Pred R-Squared" value of 0.7207 in Table 5 (response 3) is in reasonable agreement with the "Adj R-Squared" value of 0.8364, indicating a high degree of explained variance in the data. The "Adeq Precision" value of 15.270 which measures the signal-to-noise ratio, demonstrates that the model provides

Table 2: Design of experiments using 2^4 full factorial design.

Exp Run	Coded Factors				Actual Factors			
	A	B	C	D	pH	%Composition	Temp. in °C	Flow rate
1	1	1	-1	1	5	Buffer: ACN (60:40)	30	1
2	-1	-1	1	-1	4	Buffer: ACN (40:60)	50	0.8
3	1	1	-1	-1	5	Buffer: ACN (60:40)	30	0.8
4	-1	1	1	1	4	Buffer: ACN (60:40)	50	1
5	-1	1	-1	-1	4	Buffer: ACN (60:40)	30	0.8
6	1	-1	1	-1	5	Buffer: ACN (40:60)	50	0.8
7	-1	-1	-1	-1	4	Buffer: ACN (40:60)	30	0.8
8	1	-1	-1	-1	5	Buffer: ACN (40:60)	30	0.8
9	1	-1	-1	1	5	Buffer: ACN (40:60)	30	1
10	-1	-1	-1	1	4	Buffer: ACN (40:60)	30	1
11	-1	1	1	-1	4	Buffer: ACN (60:40)	50	0.8
12	1	1	1	1	5	Buffer: ACN (60:40)	50	1
13	-1	-1	1	1	4	Buffer: ACN (40:60)	50	1
14	-1	1	-1	1	4	Buffer: ACN (60:40)	30	1
15	1	-1	1	1	5	Buffer: ACN (40:60)	50	1
16	1	1	1	-1	5	Buffer: ACN (60:40)	50	0.8

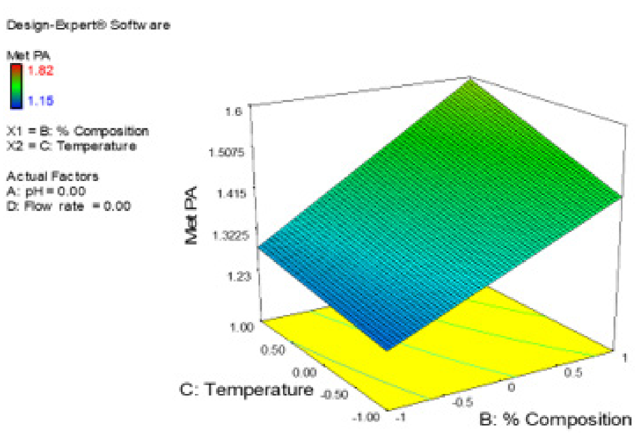

Figure 7: 3D response surface plot showing effect of B and C on Peak asymmetry of MET.

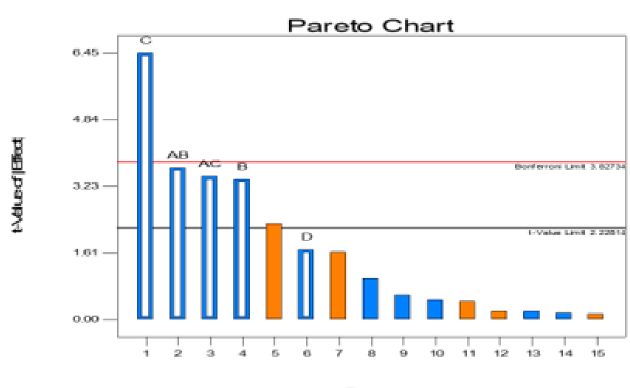

Figure 8: Pareto chart showing the effect of factors and factors interaction on peak asymmetry of TEN.

an adequate signal, as a ratio greater than 4 is desirable. Based on these results, the model can be considered appropriate for navigating the design space. Figure 8 shows the Pareto chart for effect of factor and factor interactions on PA of TEN. It indicated a decrease in factors B, C and D and interaction AB and AC decreased PA of TEN. Negative coefficient of factor B, C and D and interaction AB and AC indicated negative effect on PA of TEN. Only factors C were with significantly higher t-value than Bonferroni limit. Figure 9 shows 3D response surface plot for the effect of A and B on PA of TEN. It indicates increase in A increase PA of TEN and decrease in B decrease PA of TEN. The effect of A and B was nonlinear. Figure 10 shows 3D response surface plot for the effect of A and C on PA of TEN. It indicates increase in A increase PA of TEN and increase in C decrease PA of TEN. The effect of A and C was nonlinear.

Response 4: MAT TP

In Table 4 (d) Model F-value 39.86 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob>F" less than 0.0500 indicate model terms are significant. In this case A, B, AB are significant model terms. In Table 5 (response 4) the "Pred R-Squared" of 0.8777 is in reasonable agreement with the "Adj R-Squared" of 0.9283."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 15.618 indicates an adequate signal. This model can be used to navigate the design space. Figure 11 shows the pareto chart for effect of factor and factor interactions on TP of MET. It indicated an increase in factor B increased TP of MET. Positive coefficient of factor B indicated its positive effect on TP of MET. It indicated an increase in A, C and D factors and AB factor interaction decreased TP of MET. Negative coefficient of A, C and D factors and AB factor interaction indicated its negative effect on TP of MET. Only the factors A and C were significantly higher than Boneferroni limit. Figure 12 shows 3D response surface plot for effect of A and B on TP of MET. It indicates decrease in A and increase in B increase TP of MET. The effect of A and B was non-linear.

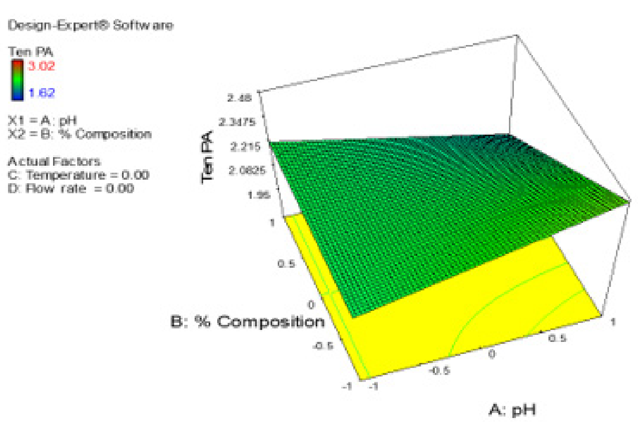

Figure 9: 3D response surface plot showing effect of A and B on Peak asymmetry of TEN.

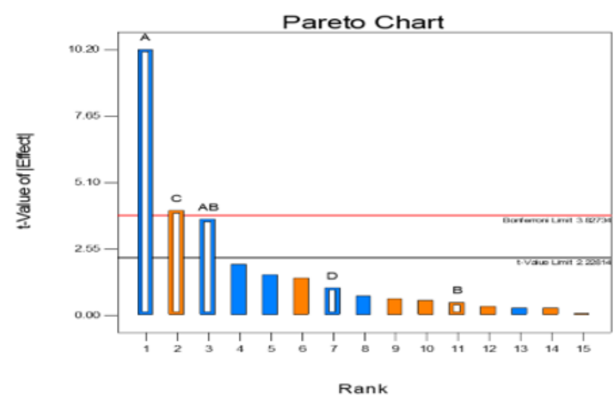

Figure 11: Pareto chart showing effects of factors and factors interaction on theoretical plate of MET.

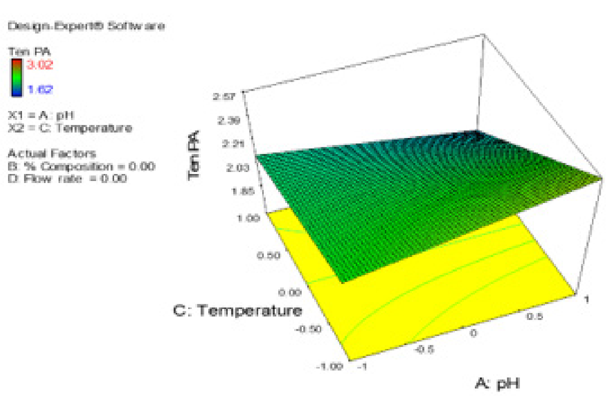

Figure 10: 3D response surface plot showing effect of A and C on Peak asymmetry of TEN.

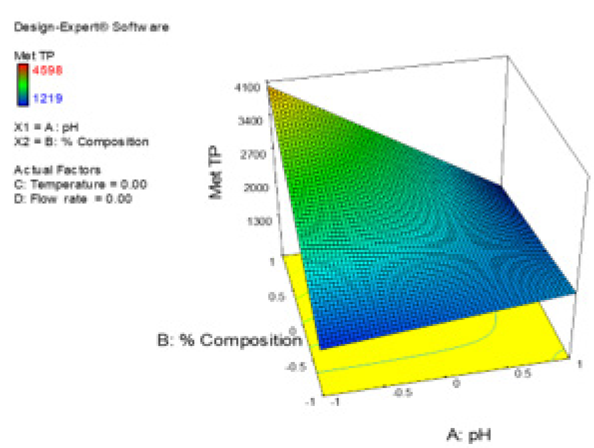

Figure 12: 3D response surface plot showing effect of A and B on MET theoretical plate.

Response 5: TEN TP

The results of the statistical analysis reveal that the Model F-Value of 27.06 as reported in Table 4 (e) is significant and suggests that the model is well-fit. The low "Prob>F" value of 0.01% supports this conclusion, indicating that there is only a minuscule chance that the Model F-Value could be attributed to random noise. The significant model terms as identified by the "Prob>F" value of less than 0.0500, include A, C and AB. The Predictive R-Squared value of 0.8238 and the Adjusted R-Squared value of 0.8968, reported in Table 5 (response 5), indicate that the model is in reasonable agreement and can be used to explore the design space. The "Adeq Precision" metric, which measures the signal to noise ratio, has a value of 15.497 and is therefore considered adequate, as a ratio greater than 4 is desirable. The Pareto chart in Figure 13 illustrates the effect of factors and factor interactions on TP of TEN. The results suggest that increasing factors B and C positively impact TP of TEN, while increasing factors A and D and the AB factor interaction have a negative effect on TP of TEN. Only A and C were found to have significantly higher t-values than the Bonferroni limit. Finally, the 3D response surface plot in Figure 14 shows the effect of A and B on TP of TEN. The plot suggests that decreasing A and increasing B result in an increase in TP of TEN. The relationship between A and B is linear.

Response 6: MET CF

The results of the statistical analysis presented in Table 4 (f) suggest that the model is statistically significant, as indicated by the large "Model F-Value" of 152.76. This value is highly unlikely to occur due to random noise, with a probability of only 0.01%. The "Prob>F" values for the model terms A, B, D and AB were less than 0.0500, indicating that these terms are statistically significant in determining the Capacity Factor (CF) of MET. Conversely, the model terms with "Prob>F" values greater than 0.1000 were deemed not statistically significant. Table 5 (response 6) displays the goodness of fit of the model, as indicated by the "Pred R-Squared" value of 0.9669 and the "Adj R-Squared" value of 0.9806. The "Adeq Precision" value of 38.152, which measures the signal-to-noise ratio, indicates an adequate signal and suggests that this model can effectively navigate the design space. The Pareto chart presented in Figure 15 provides insight

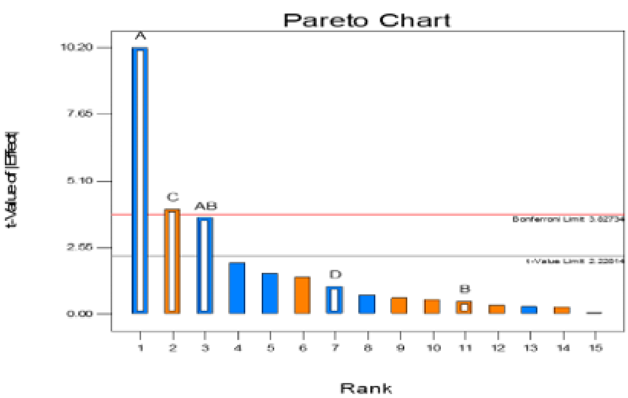

Figure 13: Pareto chart showing effects of factors and factors interaction on theoretical plate of TEN.

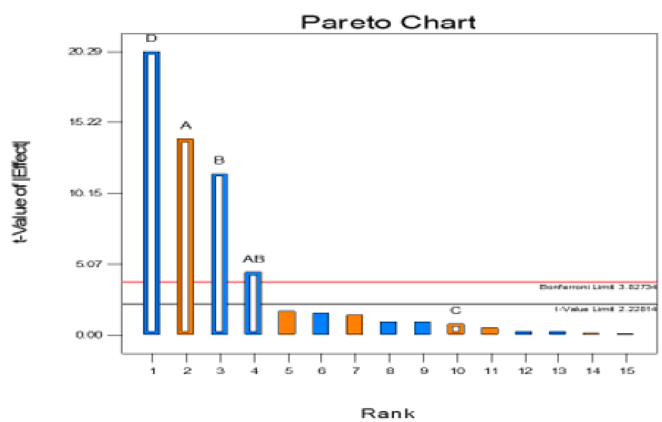

Figure 15: Pareto chart showing effects of factors and factors interaction on capacity factor of MET.

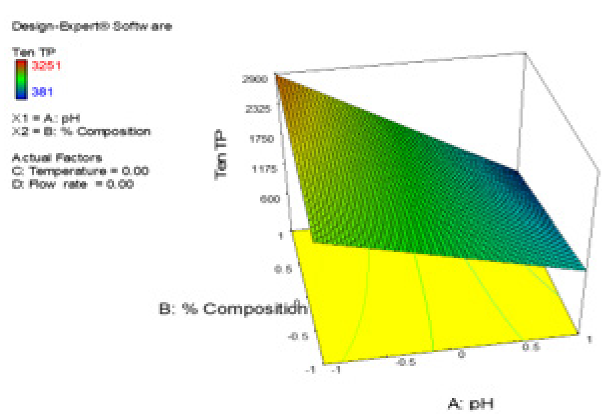

Figure 14: 3D response surface plot showing effect of A and B on theoretical plate of TEN.

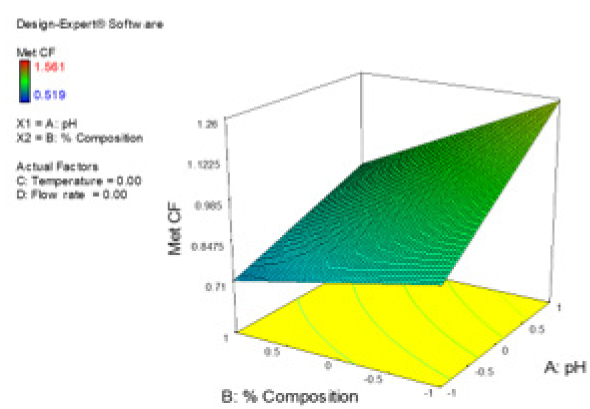

Figure 16: 3D response surface plot showing effect of A and B on capacity factor of MET.

Table 3: Experimental runs and responses measured as 2^4 full factorial design.

Run	Factors				Responses						
	A	B	C	D	Rs	CF MET	CF TEN	PA MET	PA TEN	TP MET	TP TEN
1	1	1	-1	1	4.90	0.703	2.963	1.27	2.16	1471	450
2	-1	-1	1	-1	2.90	1.061	1.686	1.25	1.13	1348	2697
3	1	1	-1	-1	4.85	1.116	3.938	1.37	2.14	1392	441
4	-1	1	1	1	6.26	0.519	1.387	1.71	2.09	3421	3010
5	-1	1	-1	-1	6.58	0.912	2.048	1.62	2.38	4598	2743
6	1	-1	1	-1	3.20	1.561	2.387	1.35	1.96	2276	2007
7	-1	-1	-1	-1	3.03	1.078	1.771	1.16	2.50	1512	2014
8	1	-1	-1	-1	3.07	1.449	3.084	1.31	3.02	1593	397
9	1	-1	-1	1	3.02	0.963	2.290	1.31	2.87	1431	381
10	-1	-1	-1	1	3.01	0.666	1.222	1.15	2.21	1488	2021
11	-1	1	1	-1	6.65	0.892	1.980	1.82	2.23	4060	3251
12	1	1	1	1	5.38	0.709	2.672	1.40	1.62	1242	747
13	-1	-1	1	1	2.69	0.646	1.155	1.20	2.05	1219	2057
14	-1	1	-1	1	6.29	0.536	1.451	1.54	2.27	3998	2538
15	1	-1	1	1	2.94	1.042	1.694	1.33	1.93	1949	1726
16	1	1	1	-1	5.43	1.133	3.559	1.45	1.82	1229	776

Where, Rs=Resolution, CF=Capacity factor, PA=Peak asymmetry and TP=Theoretical plate.

Table 4: ANOVA table for selected factorial model.

Table 4 (a): Response 1 (Resolution).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	35.16	5	7.03	181.79	< 0.0001	Significant
A-pH	1.33	1	1.33	34.49	0.0002	
B-% Composition	31.58	1	31.58	816.56	< 0.0001	
C-temperature	0.031	1	0.031	0.79	0.3945	
D-Flow rate	0.093	1	0.093	2.40	0.1520	
AB	2.12	1	2.12	54.73	<0.0001	
Residual	0.39	10	0.039			
Cor total	35.55	15				

Table 4 (b): Response 2 (MET PA).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	0.56	6	0.093	83.52	<0.0001	Significant
A-pH	0.027	1	0.027	24.56	0.0008	
B-% Composition	0.28	1	0.28	253.44	<0.0001	
C –Temperature	0.038	1	0.038	34.31	0.0002	
D-Flow rate	0.011	1	0.011	9.95	0.0117	
AB	0.19	1	0.19	170.73	<0.0001	
BC	9.025E-003	1	9.025E-003	8.14	0.0190	
Residual	9.975E-003	9	1.108E-003			
Cor Total	0.57	15				

Table 4 (c): Response 3 (TEN PA).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	1.70	5	0.34	16.33	0.0002	Significant
B-% Composition	0.24	1	0.24	11.56	0.0068	
C-Temperature	0.86	1	0.86	41.65	<0.0001	
D-Flow rate	0.060	1	0.060	2.89	0.1199	
AB	0.28	1	0.28	13.53	0.0043	
AC	0.25	1	0.25	12.04	0.0060	
Residual	0.21	10	0.021			
Cor Total	1.90	15				

Table 4 (d): Response 4 (MET TP).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	1.963E+007	5	3.926E+006	39.86	<0.0001	Significant
A-pH	5.131E+006	1	5.131E+006	52.09	<0.0001	
B-%Composition	4.617E+006	1	4.617E+006	46.87	<0.0001	
C-Temperature	34132.56	1	34132.56	0.35	0.5692	
D-Flow rate	2.000E+005	1	2.000E+005	2.03	0.1846	
AB	9.649E+006	1	9.649E+006	97.95	<0.0001	
Residual	9.851E+005	10	98509.01			
Cor Total	2.062E+007	15				

Table 4 (e): Response 5 (TEN TP).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	1.460E+007	5	2.920E+006	27.06	<0.0001	Significant
A-pH	1.123E+007	1	1.123E+006	104.10	<0.0001	
B-%Composition	26896.00	1	26896.00	0.25	0.6284	
C-Temperature	1.746E+006	1	1.746E+006	16.19	0.0024	
D-Flow rate	1.218E+005	1	1.218E+005	1.13	0.3130	
AB	1.470E+006	1	1.470E+006	13.63	0.0042	
Residual	1.079E+006	10	1.079E+005			
Cor Total	1.568+007	15				

Table 4 (f): Response 6 (MET CF).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	1.35	5	0.27	152.76	<0.0001	Significant
A-pH	0.35	1	0.35	197.34	<0.0001	
B-%Composition	0.24	1	0.24	133.49	<0.0001	
C-Temperature	1.225E-003	1	1.225E-003	0.69	0.4253	
D-Flow rate	0.73	1	0.73	411.83	<0.0001	
AB	0.036	1	0.036	20.47	0.0011	
Residual	0.018	10	1.773E-003			
Cor Total	1.37	15				

Table 4 (g): Response 7 (TEN CF).

Source	Sum of Square	Degree of freedom	Mean square	F value	*p* value Prob>F	
Model	10.22	5	2.04	63.65	<0.0001	Significant
A-pH	6.11	1	6.11	190.22	<0.0001	
B-%Composition	1.39	1	1.39	43.15	<0.0001	
C-Temperature	0.32	1	0.32	9.83	0.0106	
D-Flow rate	1.97	1	1.97	61.44	<0.0001	
AB	0.44	1	0.44	13.61	0.0042	
Residual	0.32	10	0.032			
Cor Total	10.54	15				

Table 5: Summary statistics for responses.

Response	1	2	3	4	5	6	7
	Resolution	MET PA	TEN PA	MET TP	TEN TP	MET CF	TEN CF
SD	0.20	0.033	0.14	313.16	328.48	0.042	0.18
Mean	4.39	1.39	2.21	2139.19	1703.50	0.94	2.21
C.V%	4.48	2.40	6.52	14.19	19.28	4.50	8.13
PRESS	0.99	0.032	0.53	2.522E+006	2.762E+006	0.045	0.82
R-Squared	0.9891	0.9824	0.8909	0.9522	0.9312	0.9871	0.9695
Adj R-Square	0.9837	0.9706	0.8364	0.9283	0.8968	0.9806	0.9543
Pred R-Square	0.9721	0.9442	0.7207	0.8777	0.8238	0.9669	0.9220
Adeq Precision	31.365	28.723	15.270	15.618	15.497	38.152	25.584

Table 6: Applying constraints to the design.

Name	Goal	Lower Limit	Upper Limit	Lower weight	Upper Weight	Importance
pH	is in range	-1	1	1	1	3
%Composition	is in range	-1	-1	1	1	3
Temperature	is equal to-1	-1	-1	1	1	3
Flow rate	is equal to 1	1	-1	1	1	3
Resolution	is in range	2.69	6.65	1	1	3
Met PA	is in range	1.15	1.82	1	1	1
Ten PA	Minimize	1.62	3.02	1	1	3
Met TP	is in range	1219	4598	1	1	1
Ten TP	Maximize	381	3251	1	1	3
Met CF	is in range	0.519	1.561	1	1	1

Table 7: Optimized method according to design of experiment.

Sl. No.	pH	%Com.	Temp.	Flow rate	RS	MET TP	TEN TP	MET CF	Desirability
1.	-1	0.64	-1	1	5.68	3480.96	2344	0.518	0.608
2.	-0.097	0.69	-1	1	5.75	3509.68	2328	0.518	0.605

Table 8: Linearity of MET.

Sl. No.	Concentration (µg/mL)	Peak Area (mAU*min)
1.	50	61.250
2.	100	116.283
3.	150	167.87
4.	200	211.69
5.	250	252.71
6.	300	289.25

into the effect of individual factors and factor interactions on the CF of MET. The chart indicates that an increase in factors A and C results in an increase in CF of MET, while an increase in B and D and the AB interaction has a negative effect on CF. Only factors A, B, D and the interaction AB were found to be significantly higher than the Bonferroni limit. The 3D response surface plot in Figure 16 further illustrates the effect of factors A and B on CF. The plot shows that an increase in factor A leads to an increase in CF, while an increase in factor B results in a decrease in CF. The relationship between A and B was determined to be linear.

Response 7: TEN CF

The results presented in Table 4 (g) demonstrate the significance of the model through the Model F-value of 63.65, which has a probability of only 0.01% of occurring as a result of random noise. The "Prob>F" values, which must be less than 0.0500 to indicate significance, support this finding by showing that the model terms A, B, C, D and AB are significant. Table 5 (response 7) further supports the validity of the model through its "Pred R-Squared" and "Adj R-Squared" values of 0.9220 and 0.9543 respectively, which show a reasonable level of agreement. The "Adeq Precision" value of 25.584, which measures the signal to noise ratio, indicates an adequate signal with a ratio greater than 4, allowing for the utilization of the model in navigating the design space. The Pareto chart shown in Figure 17 displays the effect of factors and factor interactions on the Capacity Factor (CF) of TEN. The chart shows that the CF of TEN is increased by the factors A and B, as well as the interaction AB, as indicated by their positive coefficients. In contrast, the CF of TEN is decreased by the factors C and D, as indicated by their negative coefficients. Only the factors A, B and D have coefficients significantly higher than the Bonferroni limit. The 3D response surface plot shown in Figure 18 further highlights the effect of factors A and B on the CF of TEN, showing a linear increase in CF as both factors increase. These results demonstrate the important role that factors A and B play in the optimization of the CF of TEN.

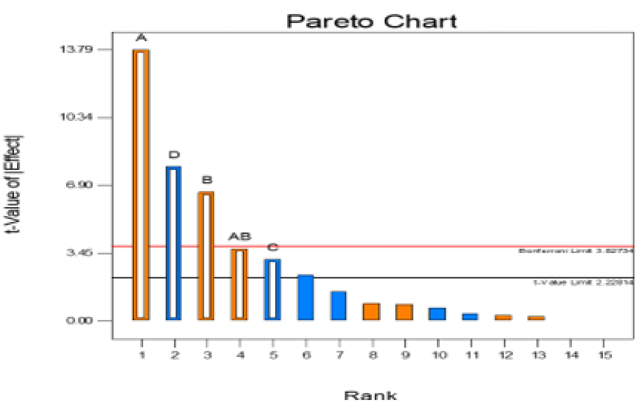

Figure 17: Pareto chart showing effects of factors and factors interaction on capacity factor of TEN.

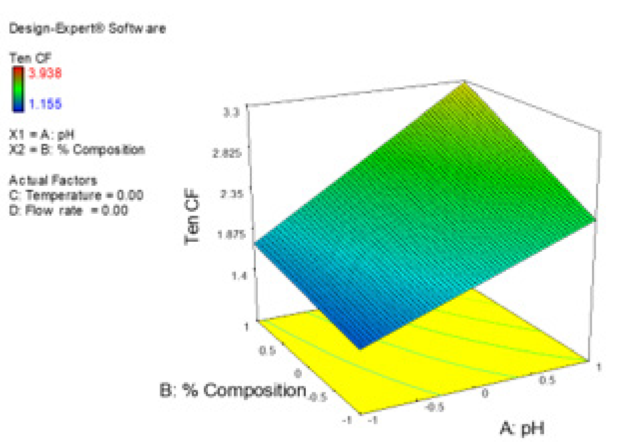

Figure 18: 3D response surface plot showing effect of A and B on capacity factor of TEN.

Polynomial equations for the response generated by ANOVA are mention below:

Resolution=+4.39-0.29*A+1.41*B+0.044*C-0.076*D-0.36*A*B

MET peak asymmetry=+1.39-0.041*A+0.13*B+0.04 9*C-0.026*D-0.11*A*B+0.024*B*C

TEN peak asymmetry=+2.21-0.12*B-0.23*C-0.06 1*D-0.13*A*B-0.12*A*C

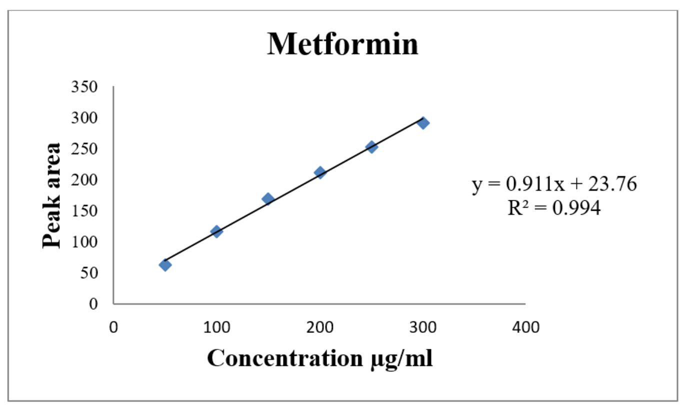

Figure 19: Calibration curve of MET at 250 nM (50-300 µg/mL).

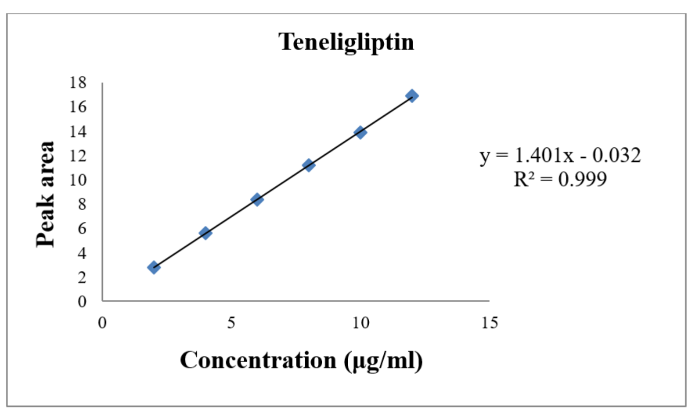

Figure 20: Calibration curve of TEN at 250 nm (2-12µg/mL).

Table 9: Linearity of TEN.

Sl. No.	Concentration (μg/mL)	Peak Area (mAU*min)
1.	2	2.80
2.	4	5.60
3.	6	8.349
4.	8	11.157
5.	10	13.87
6.	12	16.90

Table 10: Result of Accuracy study (*n*=3).

Drugs	Conc. Level (%)	Amount taken (μg/mL)	Amount added (μg/mL)	Total Amount (μg/mL)	% Recovered			Mean±S.D	% RSD
MET	50%	100	50	150	100.81	100.33	99.33	100.15±0.75	0.76
	100%	100	100	200	101.5	101.62	101.17	101.43± 0.23	0.23
	150%	100	150	250	100.31	100.59	101.16	100.68± 0.43	0.43
TEN	50%	4	2	6	99.68	99.58	99.2	99.48±0.01	1.10
	100%	4	4	8	99.76	99.62	98.5	99.29±0.69	0.69
	150%	4	6	10	99.2	98.58	98.5	98.76±0.38	0.38

Table 11: Result of Precision study (*n*=3).

Drug	Parameters	Intraday precision concentration (μg/mL)			Inter day precision concentration (μg/mL)		
		50	100	150	50	100	150
MET	Mean	61.19	115.68	168.82	61.19	116.07	169.24
	S.D	0.063	0.583	1.104	0.063	0.375	1.090
	%RSD	0.104	0.504	0.652	0.102	0.322	0.644

Table 12: Result of Precision study (*n*=3).

Drug	Parameters	Intraday precision concentration (μg/mL)			Inter day precision concentration (μg/mL)		
		2	4	6	2	4	6
TEN	Mean	2.203	5.553	8.2	2.16	5.59	8.42
	SD	0.015	0.050	0.1	0.01	0.050	0.07
	%RSD	0.680	0.900	1.211	0.462	0.894	0.831

Table 13: Result of Repeatability (*n*=6).

Sl. No.	MET (100 μg/mL)	TEN (4 μg/mL)
	Peak Area (mAU*min)	Peak Area (mAU*min)
1.	115.12	5.60
2.	115.28	5.62
3.	115.30	5.67
4.	115.35	5.69
5.	115.40	5.70
6.	115.50	5.75
Mean	115.325	5.671
S.D	0.127	0.054
%RSD	0.11	0.95

Table 14: Result of LOD and LOQ.

Sl. No.	Parameters	MET	TEN
1.	Mean of slope	0.9135	1.3446
2.	Standard deviation of intercepts	0.3546	0.1890
3.	LOD (μg/mL)	1.281	0.464
4.	LOQ (μg/mL)	3.881	1.407

Table 15: Result of Robustness.

Drug	Conc. found at Optimized Condition	Conc. found at flow rate 1.1 mL/min	Conc. found at flow rate 0.9 mL/min	Avg±SD	% RSD
MET	61.28	61.25	61.00	61.1765±0.153	0.25
TEN	5.62	5.54	5.54	5.53667±0.085049	1.54

Table 16: Result of assay of tablet dosage form (*n*=3).

Tenglyn M (500 mg MET+ 20 mg TEN)	Parameters	MET Concentration (100 µg/mL)	TEN Concentration (4 µg/mL)
	Mean±SD	101.79±0.4878	99.95±1.081
	%RSD	0.479	1.081

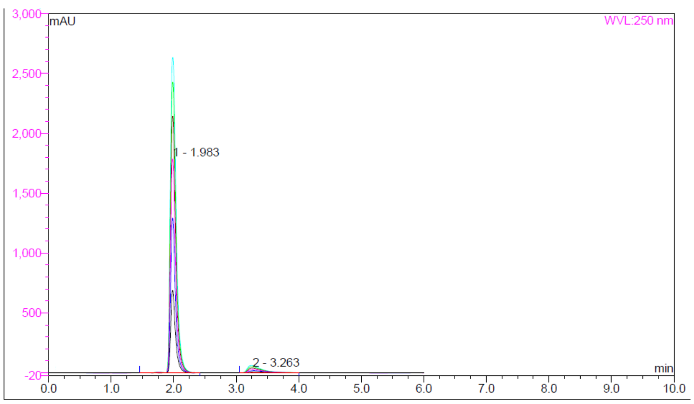

Figure 21: Overlain of Chromatograms (50-300 µg/mL for MET at retention time 1.983).

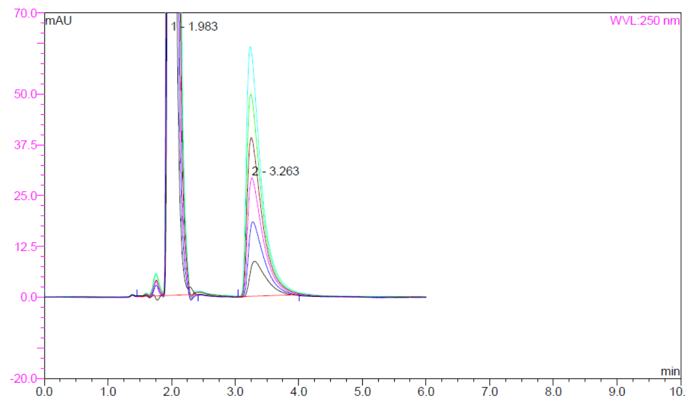

Figure 22: Overlain of Chromatograms (2-12 µg/mL for TEN at retention time 3.263).

MET theoretical plate=+2139.19-566.31*A+537.19*B-46.19*C-111.81*D-776.56*A*B

TEN theoretical plate=+1703.50-837.88*A+41.00*B+330.38*C-87.25*D-303.13*A*B

MET capacity factor=+0.94+0.15*A-0.12*B+8.750E-003*C-0.21*D-0.048*A*B

TEN capacity factor=+2.21+0.62*A+0.29*B-0.14*C-0.35*D+0.17*A*B

In order to determine optimized chromatographic conditions from the design, various constraints were applied to the design which are shown in Table 6. The solution offered by the software is shown in Table 7.

Method validation

Linearity

For both the drugs, linearity study was carried out at six different concentration levels. The linearity of MET and TEN was found in the range of 50-300 µg/mL and 2-12 µg/mL respectively. Peak areas obtained with the respective concentrations in µg/mL are shown in Table 8 and Table 9 for MET and TEN respectively. Calibration curves and chromatogram of MET and TEN were shown in Figures 19-20 and Figures 21-22 respectively.

Accuracy

The standard addition method was used to do the accuracy study. The % RSD was found to be between 0.23 and 0.76 for MET and between 0.38 and 1.10 for TEN. This showed that the method was accurate. Table 10 shows results of recovery study.

Intermediate precision

The values of % RSD for MET were found to be between 0.104-0.652 for intra-day precision and between 0.102-0.644 for inter-day precision. The intra-day and inter-day precision, % RSD values for TEN were found to be between 0.680-1.211 and 0.462-0.894, respectively. Tables 11 and 12 show the results of the precision study, which showed the precised method.

Repeatability

In repeatability study % RSD for MET and TEN was found to be 0.11 and 0.95, which can be seen in Table 13.

LOD and LOQ

The LOD value for MET was determined to be 1.281 μg/mL, whereas the LOD value for TEN was 0.464 μg/mL. For MET and TEN, LOQ were determined to be 3.881 μg/mL and 1.407 μg/mL respectively. Results of LOD and LOQ of MET and TEN are shown in Table 14.

Robustness

The method was determined to be robust because the results were not significantly impacted by minor variations in pH, composition of mobile phase, temperature and flow rate as determined by experimental design runs. Table 15 displays the changes in flow rate that were observed.

Analysis of tablet dosage form

The proposed RP-HPLC method for the determination of MET and TEN in tablet dosage form was successfully applied. It was determined that the percentage of MET and TEN was satisfactory and comparable to the label claim (Table 16).

DISCUSSION

Based on a review of the relevant literature, the C18 column was chosen for the study. The mobile phase was allowed to saturate the column. To achieve the best separation of MET and TEN, various mobile phases comprised of methanol, acetonitrile, water and ammonium acetate buffer were tested at various flow rates and compositions. At a detection wavelength of 250 nm, detector responses for drugs were significantly enhanced. In terms of resolution and peak shape, the mixture of ammonium acetate buffer: acetonitrile (56.4:43.6 V/V) at a flow rate of 1.0 mL/min and a temperature of 30°C proved to be superior to the other mixtures.

CONCLUSION

The method was successfully developed and optimized using experimental design. Using ANOVA, the significant effect of independent factors was analysed. It was determined that the proposed method was accurate, precise and robust. Therefore, it is suitable for routine analysis of MET and TEN in pharmaceutical dosage forms.

ACKNOWLEDGEMENT

The authors are thankful to Faculty of Pharmacy, Dharmsinh Desai University for providing facilities to carry out the work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RP-HPLC: Reversed phase high performance liquid chromatography; **QbD:** Quality by design; **DOE:** Design of experiment; **LOD:** Limit of detection; **LOQ:** Limit of Quantification; **MET:** Metformin; **TEN:** Teneligliptin; **AMPK:** Adenosine Monophosphate Activated Protein Kinase; **DPP:** Dipeptidyl peptidase; **GLP:** Glucagon like peptide; **HPTLC:** High-performance thin layer chromatography; **RS:** Resolution; **CF:** Capacity factor; **PA:** Peak asymmetry; **TP:** Theoretical plates; **ICH:** International conference on harmonization.

SUMMARY

Many researchers conclude that MET with combination of TEN have higher efficacy and therapeutic effect. So, simultaneous estimation of the drug in dosage form is an important parameter for the treatment of type 2 diabetes mellitus. This paper presented the simultaneous method for the estimation of the MET with combination of TEN through the RP-HPLC method. The concept of Quality by Design has also been utilized for making a robust simultaneous estimation of both drugs. Pareto chart and 3D response surface plot has been plotted for showing the relationship between independent and dependent variables. Outlay plot has been plotted and model of QbD has been validated by comparing the results with the experimental results. Various standard parameters as per ICH guidelines such as LOD, LOQ, Accuracy, Precision, Robustness, Repeatability has been applied for the validating the method for simultaneous estimation of MET and TEN. This research can be applied for the robust method validation of various other drugs by applying the concept of QbD.

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Cite this article: Upadhyay J, Padhiyar S, Jivani K, Patel T, Bhavsar V, Prajapati V. Quality by Design (QbD) Based Development and Validation of RP-HPLC Method for Simultaneous Estimation of Metformin and Teneligliptin in Bulk and their Pharmaceutical Formulation. Indian J of Pharmaceutical Education and Research. 2025;59(2s):s689-s704.