AQbD-Assisted Development and Validation of RP-HPLC Method for Simultaneous Quantification of Remogliflozin Etabonate, Vildagliptin, and Metformin Hydrochloride in Single Formulation

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KEYWORDS

AQbD, Diabetes, Metformin. RP-HPLC, Vildagliptin

ABSTRACT:

Introduction:

The study aimed to optimize and validate a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the simultaneous quantification of Remogliflozin Etabonate (REM), Vildagliptin (VIL), and Metformin Hydrochloride (MET) in a single pharmaceutical formulation. An Analytical Quality by Design (AQbD) approach was applied to ensure method robustness and reliability.

Objectives:

The study aimed to identify critical parameters influencing chromatographic separation, optimize method conditions using a Box-Behnken Design (BBD), and validate the RP-HPLC method in terms of retention time, linearity, and predictive accuracy.

Methods:

A risk estimate matrix was used to identify critical parameters, including acetonitrile quantity, pH, and flow rate. BBD was employed to assess their impact, and multiple linear regression models were developed to understand variable relationships. Chromatographic separation was performed using a Phenyl column at 25°C, with UV detection at 210 nm and 230 nm. The Method Operable Design Region (MODR) was determined to optimize conditions, and method validation included evaluating linearity and retention times.

Results:

The optimized method achieved efficient separation, with retention times of 4.5, 8.5, and 15.5 minutes for Vildagliptin, Remogliflozin Etabonate, and Metformin Hydrochloride, respectively. Linearity was established for Vildagliptin (1.25–37.5 µg/mL), Remogliflozin Etabonate (2.5–75 µg/mL), and Metformin Hydrochloride (25–750 µg/mL).

Conclusions:

The AQbD-based RP-HPLC method was successfully optimized and validated, ensuring accurate, reliable, and efficient simultaneous quantification of the three analytes in pharmaceutical formulations.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a persistent and advancing medical illness that presents an increasing public health issue on a global scale. It is treated with many non-insulin antidiabetic medications (NIADs) that focus on managing high blood sugar levels [1]. The majority of cases of diabetes mellitus eventually need the use of a combination of medications in order to provide an enhanced antihyperglycemic impact, while minimizing the potential side effects associated with each pharmacological class [2]. Currently, the most often recommended medicine for the treatment of

T2DM is a mix of newer treatments. This specific medicine combination is recommended for the treatment of Type 2 diabetes. The triple fixed dose combination of Remogliflozin Etabonate (REM; 100 mg), Vildagliptin (VIL; 50 mg), and Metformin Hydrochloride (MET; 1000 mg) is frequently prescribed to individuals with T2DM as safe, well tolerated and effective regimen [1]. This medication should be taken twice daily to enhance glycaemic control.

Remogliflozin Etabonate is a prodrug of REM, 5-methyl-1-(propan-2-yl).4-(propan-2-yloxy) benzyl, also known as -4-[4-(propan-2-yloxy) ben. The compound is 1H-pyrazol-3-yl 6-O-(ethoxycarbonyl). It hinders the activity of sodium-glucose transport proteins, which have a role in the reabsorption of glucose in the kidney [3, 4]. Vildagliptin is a compound with the chemical formula S-1-[N-(3-hydroxy-1-adamantyl) glycyl]. These inhibitors are a recent addition to the family of oral antihyperglycemic medicines used to control high blood sugar in individuals with T2DM [5, 6]. It functions by specifically targeting the enzyme DPP-4, which is involved in breaking down incretin hormones including glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide [7]. Metformin (also known as Glucophage) came into clinical practice in 1956. Use in 10 years old children to elderly, including mild-moderate renal failure patients, apparent protection against atherosclerotic events were supported by UK Prospective diabetes study . Metformin Hydrochloride, a biguanide antidiabetic medication, is often used to regulate blood glucose levels. According to many international standards, it is widely accepted as the first therapy for T2DM [8,9]. Metformin works by decreasing hepatic glucose output, increasing intestinal glucose utilization along with increased insulin mediated peripheral glucose uptake. Figure 1 displays the chemical compositions of the suggested medications.

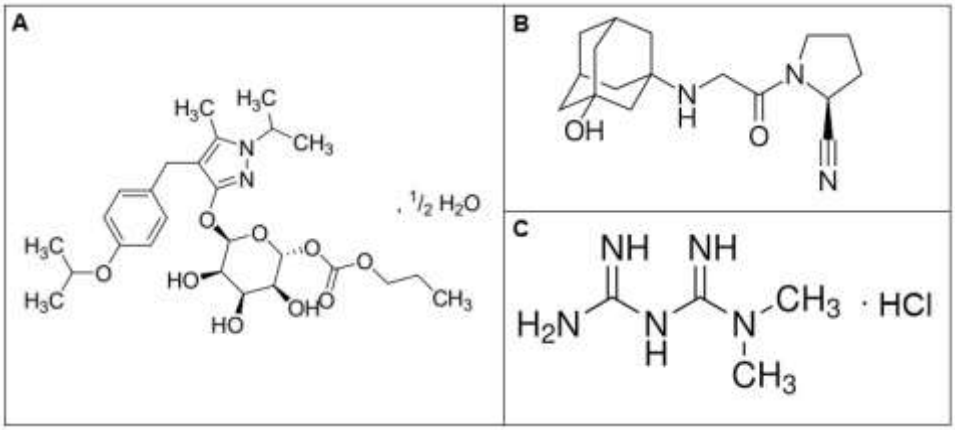

Figure 1: Chemical structures of (A) Remogliflozin Etabonate, (B) Vildagliptin (B) and (C) Metformin Hydrochloride

Regarding the introduction of the revised Q14 guideline by the International Council for Harmonisation (ICH) in June 2018, the concept of Analytical Quality by Design (AQbD) has been established for the creation and validation of analytical procedures. The AQbD technique helps to establish an effective control plan for analytical processes, ensuring that sources of variability are managed and consistent, high-quality findings are consistently obtained [10]. Unlike the old strategy that focuses on optimizing one factor at a time (OFAT), the new AQbD approach prioritizes science and risk-based QbD principles. This approach enables method development by efficiently using resources [11,12]. The creation of methods based on Analytical Quality by Design (AQbD) offers advantages such as enhanced method resilience, resulting in fewer unexpected mistakes and out-of-specification (OOS) results. This leads to a reduction in the number of further revisions required after approval, resulting in a cost-effective approach with enhanced confidence of analytical findings [13]. The Box-Behnken design (BBD) is a second-order three-level factorial design that is more efficient

in optimization compared to other designs such as complete factorial, central composite, d-optimal, and mixed design [14]. The design is sequential and utilizes a viable design matrix to display the quadratic model. It also provides a clear evaluation of CMAs. The experimental design must adhere to a particular quadratic model's statistics or lack of fit values, which necessitates the use of just three coded levels: low (-1), medium (0), and high (+1).

Acharya et al., developed stability-indicating HPLC method for REM, VIL and MET using Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) with mobile phase comprising of a mixture of 50% Phosphate buffer (pH - 6.8) and 50% Acetonitrile at a flow rate of 0.8 mL/min and 20 minutes of run time [15]. Similarly, Sethy et al., developed RP- HPLC method which used a (150 × 4.6) mm, 2.7 mm C18 column, Ascentis as stationary phase and mobile phase made up of acetonitrile (ACN) and phosphate buffer in a ratio of 35:65 (%V/V) using PDA detector [16]. However, while evaluating scientific databases, no complete analytical methodologies were found that implemented AQbD to assist in the robust and risk-free measurement of a combination of three substances: REM, VIL, and MET. Therefore, an AQbD based HPLC method was used to simultaneously determine the concentrations of REM, VIL, and MET in a single dose form. Quality Risk Management (QRM) was conducted by identifying possible risk factors in the development of the chromatographic technique and analysing their level of risk using the Risk Estimation Matrix (REM), in accordance with the ICH Q9 standard [17,18]. This study covers the AQbD-assisted development and validation of a RP-HPLC method for the concurrent quantification of Remogliflozin Etabonate, Vildagliptin, and Metformin Hydrochloride in a singular pharmaceutical formulation. The proposed approach was developed to be robust, efficient, and in accordance with the International Council for Harmonisation (ICH) requirements for validation characteristics including accuracy, precision, specificity, linearity, and robustness.

2. Objectives

- To develop a robust and sensitive RP-HPLC method for simultaneous quantification of Remogliflozin etabonate, Vildagliptin, and Metformin HCl in single formulation
- To validate proposed method as per ICH guideline
- To employ AQbD tools for optimization of robust and reliable RP-HPLC method

3. Methods

3.1 Chemicals and reagents

The Remogliflozin Etabonate was received as a gift sample from Glenmark Pharmaceuticals Ltd., (Maharashtra, India). Vildagliptin and Metformin Hydrochloride were received as gift samples from Harman Fino chem (Aurangabad, India). The Remo-Zen MV1000 tablet was acquired from a local market in Vadodara, India. Hexane-1-sulfonic acid sodium salt (HPLC Grade), Orthophosphoric acid (HPLC Grade), Acetonitrile (HPLC Grade) were procured from M/s SD Fine chemicals, Mumbai, India. HPLC grade water (Milli-Q) was used in the study.

3.2 Instrumentation

An HPLC experiment was conducted utilizing the Waters e2695 PDA 2998 separation module and the Shimadzu i-series 2030 with an auto sampler. The separation was performed using Zorbax SB-Phenyl, 150mm X 4.6 mm, 5 µ (Agilent) or Ultisil XB Phenyl, 150mm X 4.6 mm, 5 µ (Welch) columns. The collection and analysis of data were conducted using Empower and Lab solution software. Other equipments including UV-Visible Spectrophotometer (Shimadzu Co.), Analytical Balance (Mettler Toledo), pH meter (Lab India) and Digital Ultra Sonicator (Remi) were used in the study.

3.3. Preparation of solutions

3.3.1 Diluent preparation

The diluent was produced by amalgamating 900 mL of water with 100 mL of acetonitrile in a sterile vessel and mixing thoroughly to achieve homogeneity. Subsequently, 1 mL of orthophosphoric acid was incorporated into the mixture and thoroughly agitated until a homogenous solution was achieved.

3.3.2 Preparation of working standard solution

A precisely measured amount of about 50 mg of Remogliflozin Etabonate was put into a 50 mL volumetric flask. A 30 mL solution of methanol was added and subjected to sonication in order to achieve dissolution. The solution was allowed to equilibrate to room temperature and the volume was adjusted to the desired level using methanol, ensuring thorough mixing. (Concentration of REM: 1000 µg/mL). A precisely measured amount of about 50 mg of Vildagliptin was placed into a volumetric flask with a capacity of 50 mL. A 30 mL solvent was added and subjected to sonication in order to dissolve. Permit The solution was allowed to equilibrate to room temperature and the volume was adjusted to the desired level using a diluent, then well mixed. (Concentration of VIL: about 1000 µg/mL). A precisely measured amount of about 100 mg of Metformin HCl was placed into a volumetric flask with a capacity of 200 mL. A 100 mL solvent was added and subjected to sonication in order to dissolve. The solution was let to equilibrate to ambient temperature. 10 mL of a stock solution of Remogliflozin Etabonate and 5 mL of a stock solution of Vildagliptin were combined. The volume was then adjusted to the desired level using a diluent and well mixed. The concentration of REM is about 50 µg/mL, concentration of VIL is approximately 25 µg/mL, and the concentration of MET is approximately 500 µg/mL

3.3.3 Preparation of sample solution

The process included measuring the weight and placing 5 tablets into a 1000 mL volumetric flask, followed by 700 mL of diluent and the mixture was subjected to sonication for 45 minutes, with occasional shaking. After that, the solution was allowed to reach room temperature and the volume was filled up to the designated level with diluent and well mixed. The solution was then passed through a 0.45 µ PVDF filter, with the first few millilitres of filtrate being discarded. Afterwards, 5.0 mL of the filtered sample solution was transferred to a 50 mL volumetric flask containing diluent and carefully mixed to have final concentration of 50 µg/mL (REM), 25 µg/mL (VIL) and 500 µg/mL (MET).

3.3.4 Preparation of MP

A precisely measured amount of about 9.41 grams of Hexane-1-sulfonic acid sodium salt was put into 1 litre of Milli-Q water. Additionally, it was subjected to sonication and thoroughly blended. The pH of this solution was adjusted to 2.0 ± 0.05 using Orthophosphoric acid. The mobile phase comprised of mixture of phase A (Water: Buffer: Acetonitrile in ratio of 62:30:8) and phase B (ACN) was supplied by gradient elution at a flow rate of 1.0 mL/min.

3.4 Application of AQbD tools and DoE

3.4.1. Analytical Target Profile (ATP) and Critical Method Attributes (CMAs)

The analytical target profile of the RP-HPLC technique for the proposed REM, VIL, and MET was determined and supported with justification. Critical method technique Attributes (CMAs) are quantifiable characteristics of the chromatogram that must fall within specified limits to guarantee the intended quality of the proposed technique. In this work, the parameters chosen as CMAs were retention duration for each medication, tailing factor, and resolution [19-21].

3.4.2 Risk assessment and preliminary experimentation

An Ishikawa diagram was created to identify and analyze possible risk factors associated with the methodology, using information from first experiments and existing understanding of chromatography. A risk assessment matrix was outlined to establish the level of risk associated with CMAs. The risk was classified into high, medium, and low levels as per their magnitude and impact on CMAs.

3.4.3. BBD assisted method optimization

After a thorough review of potential risks, the Box-Behnken Design (BBD) was applied to examine the primary, interaction, and quadratic effects of the screening variables on the chosen responses. The specific BBD matrix was as shown in Table 1. The independent variables were concentration of ACN in the mobile phase (X1), the pH of the buffer in mobile phase-A (X2), and the flow rate (X3). Dependent parameters were tailing actor (R1), retention time (R2), and resolution (R3), respectively, for REM, tailing factor (R4) and retention time (R5) for MET, tailing factor (R6) and retention time (R7) for VIL. An examination of the response surface was conducted using ANOVA and DoE plots. A significance level was evaluated when the p-value was less than 0.05.

Table 1: Experimental runs using BBD

Run	Coded values			Actual values		
	ACN concentration in MP (%)	pH	Flow Rate	ACN concentration in MP (%)	pH	Flow Rate
	X_1	X_2	X_3	X_1	X_2	X_3
T1	0	-1	1	10	1	1.5
T2	-1	1	0	5	4	1
T3	1	1	0	15	4	1
T4	1	0	-1	15	2.5	0.5
T5	0	0	0	10	2.5	1
T6	-1	0	-1	5	2.5	0.5
T7	1	0	1	15	2.5	1.5
T8	0	1	1	10	4	1.5
T9	0	1	-1	10	4	0.5
T10	-1	0	1	5	2.5	1.5
T11	0	-1	-1	10	1	0.5
T12	-1	-1	0	5	1	1
T13	1	-1	0	15	1	1
T14	0	0	0	10	2.5	1
T15	0	0	0	10	2.5	1

3.4.4. Navigation of Method Operable Design Region (MODR) and Model Validation

The Method Operable Design Region (MODR) was used to create a multidimensional space by considering method elements and settings. Additionally, it was used to build significant technique controls, such as system appropriateness. The 2D contour plots of all seven responses were superimposed, and the target area was delineated. This zone was described as having optimal operating circumstances in which the intended outcomes can be attained. Additionally, the ANOVA-guided optimal multiple linear regression (MLR) models for various CMAs were further confirmed by check point runs (CP1 and CP2) chosen from the design space. The percentage error (PE) for

check point trials was determined by applying the following formula to the expected and experimental values of replies.

%PE = [(Experimental value - Predicted value)/Experimental value] *100

3.5 Analytical method validation

The method validation to done in accordance with the guidelines outlined in ICH Q2R1. The method's specificity was confirmed by monitoring the interference from blank and placebo formulations. The system's applicability was confirmed by injecting six reference solutions and monitoring the % RSD (relative standard deviation) of the average area, tailing factor, theoretical plates, and resolution of the medicines. The method's linearity was assessed within a range of 5% to 150% of the standard concentration. The method's accuracy was assessed in terms of the repeatability of sample measurement. Both the intermediate and intraday precision was evaluated. The accuracy of the method was evaluated by injecting six solutions and recording the percentage assay and percentage relative standard deviation (% RSD). The accuracy of the method was evaluated by measuring the amount of selected medicines that were successfully retrieved in a combined dosage form at certain concentrations of 50%, 80%, 100%, and 150%. The method's robustness was confirmed by deliberately introducing slight variations in the flow rate, column oven temperature, pH of the mobile phase, and organic ratio in the chromatographic conditions. The robustness research included the reporting of the % RSD (relative standard deviation) of the area of six duplicate injections, as well as the tailing factor and % assay.

4. Results and discussion

4.1 Preliminary runs

The objective of this work was to create a new RP-HPLC method for the concurrent determination of REM, VIL and MET in their combination pharmaceutical formulation. Initially, it was noted that all three medicines exhibited varying solubility in organic solvents and different buffer solutions. After conducting many experiments, a diluent consisting of Water: Acetonitrile: Orthophosphoric acid in a ratio of 900:100:1 (%V/V/V) was selected for making the analytical solutions. Different organic solvents such as methanol, and ACN were evaluated for their suitability as the organic phase in RP-HPLC chromatography. Initial experiments involved testing potassium di-hydrogen phosphate buffer and ammonium acetate buffer at various pH levels in combination with different organic solvents to separate the three drugs. Eventually, a combination of Hexane-1-sulfonic acid sodium salt buffer with ACN was selected for effective separation, retention and resolution. Multiple experiments were performed utilizing varied ratios of organic and buffer phases (65:35, 67:33, 70:30, and 72:28, %v/v), as well as variable flow rates (0.9 and 1.0 mL/min) with different types of C-18 and C-8 columns (250×4.6 mm ×5 µm & 150×4.6 mm ×5 µm). Excellent separation of all drugs was achieved with high resolution, minimal tailing factor, and optimal peak shape using a Phenyl column (150mm X 4.6 mm, 5 µm). The mobile phase-A, composed of a mixture of Water, Buffer, and ACN in a ratio of 62:30:8, and mobile phase-B, consisting of ACN, were used in a gradient elution program lasting 20 minutes. The flow rate was determined to be 1.0 mL/min, and the column oven temperature was adjusted to 25°C. Table 2 contains the optimized chromatographic conditions, whereas Figure 2 and Figure 3 display the chromatogram of the standard and sample, respectively.

Table 2: Optimized chromatographic conditions

Column	Phenyl, 150mm X 4.6 mm, 5µ
Flow rate	1 mL/min
Column oven Temp	25°C

Sample cooler Temperature	15°C		
Injection Volume	5 µL		
Wavelength	210 nm (VIL), 230 nm (MET & REM)		
Retention time (Min)	8.5±0.3(VIL), 4.5 ± 0.5 (MET), 15.0±0.6(REM)		
Mobile phase-A	Hexane-1-sulfonic acid sodium salt at pH 2.0		
Mobile Phase-B	Acetonitrile		
Run time	(Gradient program)		
	Time (minutes)	% Mobile phase (A)	% Mobile phase (B)
	0.01	92	08
	4.0	92	08
	12.0	50	50
	13.0	50	50
	14.0	92	08
	18.0	92	08
	20.0	92	08

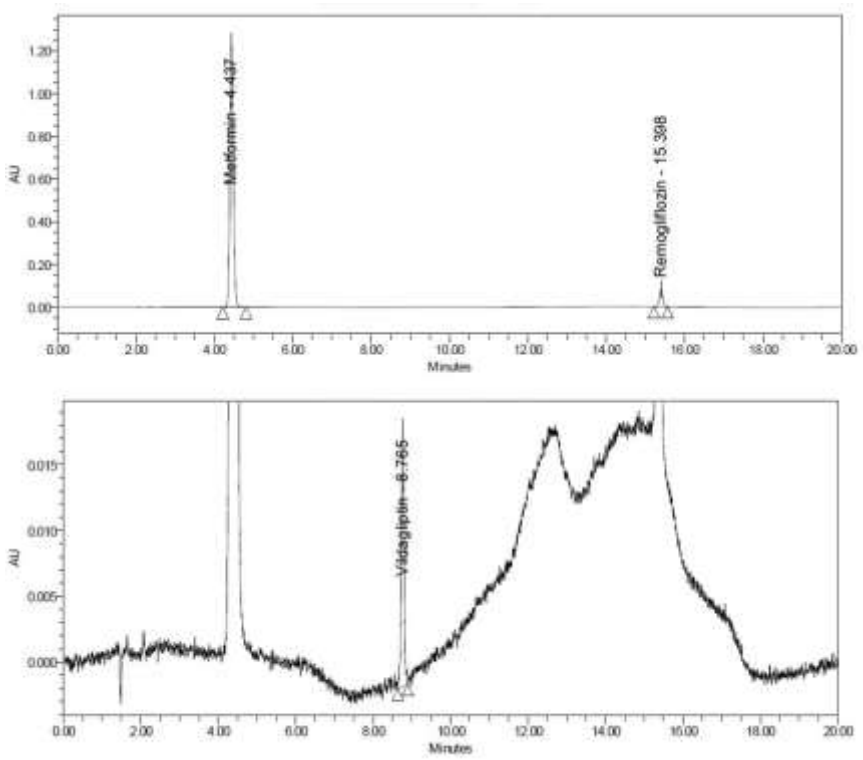

Figure 2: Chromatogram of MET, REM and VIL (Standard)

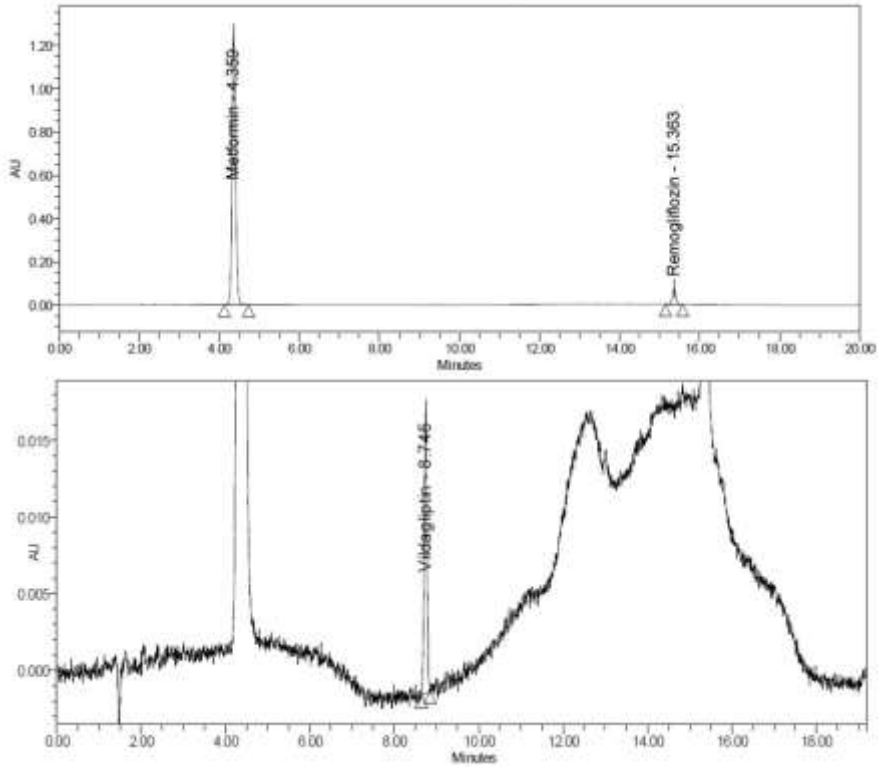

Figure 3: Chromatogram of MET, REM and VIL (Sample)

4.2 Analytical target profile (ATP) and critical method quality attributes (CMAs)

The HPLC method for detecting REM, VIL, and MET must possess high sensitivity, selectivity, speed, cost-effectiveness, and robustness and it must provide an assay result within the range of 98-102%. The summary of CMAs for proposed HPLC method is given in Table 3.

Table 3: Critical Method Attributes (CMAs)

Method attributes (MA) of the drug products	Target	Is this a CMA?	Justification
Retention time	Optimum	Yes	It must be optimally minimum to have fast detection of drug.
Peak height	Optimum	Yes*	It helps in peak symmetry for better quantification
Tailing factor	NMT 2	Yes	For better resolution from different peak with better quantification
Theoretical plates	>1500	Yes*	For better quantification
Capacity factor	>2	Yes*	It helps for quantification. But if first three CMAs are optimized in range it will be in acceptable range.
Resolution	>2	Yes	To get distinct peaks effectively
Peak area	Optimum	No	It doesn't impact on quality of method.

4.3 Risk assessment

The identified method risk parameters were organized and presented in a fishbone diagram for convenient examination in order to facilitate further risk management (Figure 4). The six primary potential sources of risk (Material, Method, Measurement, Machine, Men, Milieu) were further categorized and subcategorized in the fishbone diagram to comprehend the possible risks associated with CMAs.

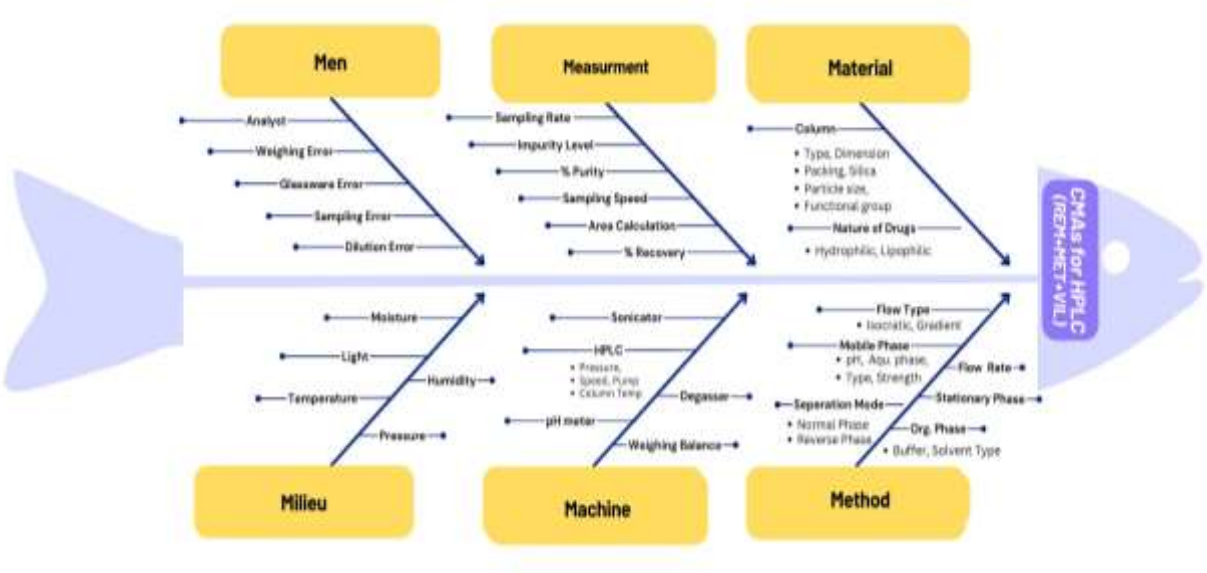

Figure 4: Fishbone diagram relating risk of factors on selected CMAs

By observing each method risk parameter listed in the fishbone diagram, a risk estimation matrix (REM) was outlined relating magnitude of risk on critical method attributes (CMAs). The risk categorized into high, medium and low values and assigned to each factor accordingly. The REM is outlined in Table 4. The REM clearly indicates mobile phase composition, pH of mobile phase and flow rate as risk factors which have high impact on selected CMAs. Column temperature was considered as factor having medium magnitude on CMAs. Column length, wavelength, injection volume and sample temperature were categorized as low risk factors. So, in further step of optimization, only highly risky factors were focused to check their impact on CMAs.

Table 4: Risk estimation matrix

CMAs	Mobile Phase	pH	CL	WL	CT	FR	IV	ST
TF	High	High	Low	Low	Medium	High	Low	Low
RT	High	High	Low	Low	Medium	High	Low	Low
Resolution	High	High	Low	Low	Medium	High	Low	Low

(TF: Tailing Factor, RT: Retention time, CL: Column Length, WL: Wave length, CT: Column Temperature, FR: Flow Rate, IV: Injection volume, ST: Sample temperature)

4.4 Optimization by BBD

The results for selected CMAs of developed HPLC method are shown in Table 5. The range of response values (R1-R7) were found to 0.8-1.5, 11.9-15, 25.9-80.4, 0.9-1.4, 1.3-5.5, 0.9-1.8 and 2.9-

13.6, respectively. The response values of BBD run present wide variations which indicates that the selected factors have strong impact on CMAs.

Table 5: Response values of the Box- Behnken design runs

Run	Responses (REM)			Responses (MET)		Responses (VIL)	
	R1	R2	R3	R4	R5	R6	R7
	TF	RT (Min)	RS	TF	RT (Min)	TF	RT (Min)
T1	1.4	12.58	80.4	0.9	1.3	1.6	3.2
T2	0.9	14.6	59.8	1.3	3.4	1.1	9.2
T3	1.4	13.3	62.6	1.4	2.2	1.7	6.2
T4	1.5	17.5	51.2	1.2	4.6	1.8	8.5
T5	1.4	14.23	69.2	1	2.9	1.6	7.6
T6	1.2	12.9	46.12	1.4	1.8	1.4	13.6
T7	1.1	11.9	71.7	1	1.4	1.2	2.9
T8	0.8	12.6	76	1.1	1.5	0.9	5.2
T9	1.4	15	27.1	1.2	5.5	1.6	11.8
T10	1.5	13.7	52	1.2	3.3	1.7	8.1
T11	1	14.3	25.9	1.2	3.9	1.1	9.3
T12	1.3	14.8	62.7	1.4	3.5	1.5	8.7
T13	1.2	13.4	78.6	1	1.7	1.4	3.14
T14	1.4	14.23	69.2	1	2.9	1.6	7.6
T15	1.4	14.25	70.3	1	2.9	1.5	7.6

ANOVA analysis of the responses R1-R7 indicates the suitable fit model for all responses and impact of individual terms (main effect, interaction and polynomial) on particular response. This assisted in derivation of mathematical models for all responses. The prediction power was increased by retaining significant terms and omitting non-significant terms. The significance level was set at 95% confidence interval (p value < 0.05). Moreover, the co-efficient values and its sign were further considered to assess the magnitude of effects on response. Table 6 depicts derived mathematical models which were used to predict the values of responses at different experimental conditions lies in design space.

Table 6: Derived mathematical models with best fit model for responses

Response	Mathematical Model	Best fit model
R1 (TF-REM)	$=1.4+0.038*A-0.05*B-0.038*C+0.15*AB-0.175*AC-0.25*BC-0.0125*A^2-0.187*B^2-0.0625*C^2$	Quadratic (p = 0.0084)
R2 (RT-	=13.95+0.013*A+0.052*B-1.115*C+0.025*AB-1.6*AC-0.17*BC	2FI

REM)		(*p* = 0.0134)
R3 (RS-REM)	=60.19+5.435*A-2.76*B+16.22*C	Linear (*p* = 0.00154)
R4 (TF-VIL)	=1.00-0.088*A+0.063*B-0.1*C+0.125*AB+0.05*BC+0.188*A^2+0.087*B^2+ 0.0125*C^2	Quadratic (*p* = < 0.001)
R5 (RT-VIL)	=2.85-0.263*A+0.275*B-1.0375*C+0.15*AB-1.175*AC-0.35*BC	2FI (*p* = 0.040)
R6 (TF-MET)	=1.567+0.05*A-0.037*B-0.062*C+0.175*AB-0.225*AC-0.3*BC+0.041*A^2-0.183*B^2-0.083*C^2	Quadratic (*p* = 0.012)
R7 (RT-MET)	=7.6-2.36*A+1.01*B-2.975*C+0.64*AB-0.025*AC-0.125*BC+0.055*A^2-0.845*B^2+0.62*C^2	Quadratic (*p* = < 0.001)

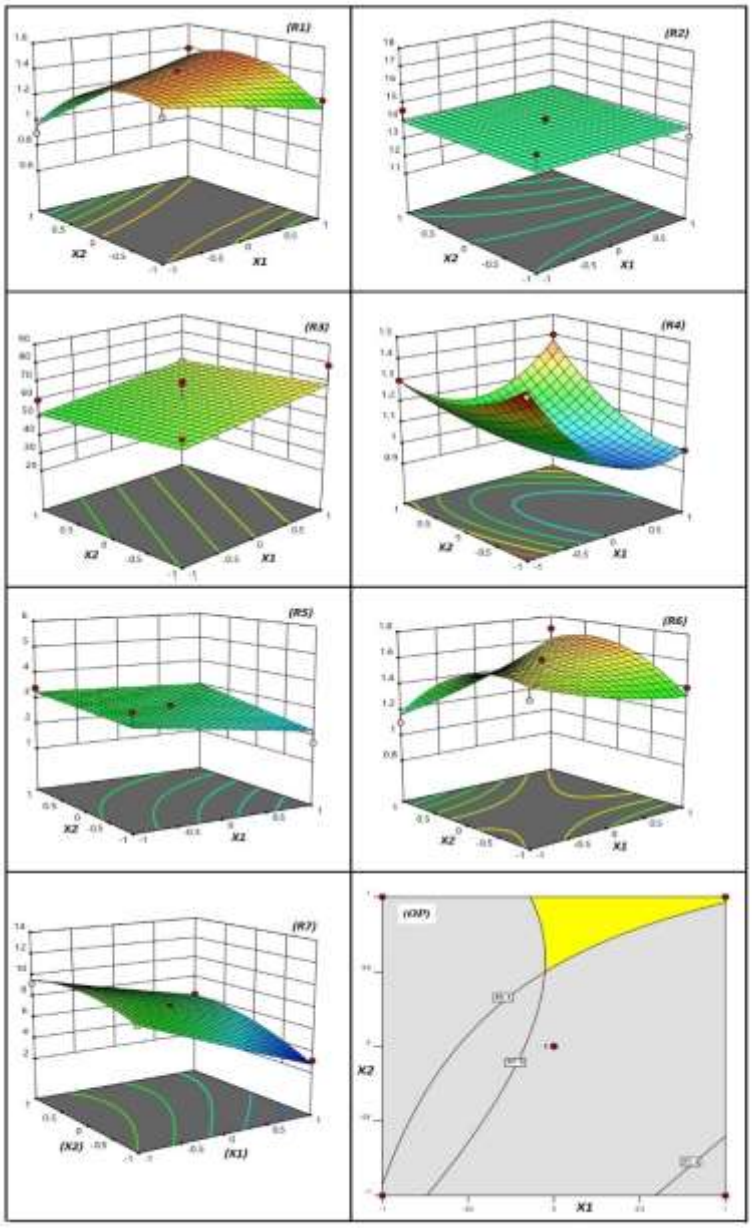

Figure 5: 3D response surface plots for responses (R1-R7) and overlay plot

3.5 Navigation of method operable design region (MODR) and model validation

To outline the method operatable, deign region for HPLC method, all 2D contour plots of all responses were overlapped. The region shown in yellow color in Figure 5 indicates design space which covered the desired ranges of responses. In order to verify the accuracy of the created models for various CMAs of the HPLC technique, specific test runs (CP1 and CP2) were selected from the design space (Figure 6). The checkpoint batches were chosen from the yellow area. The experimental runs were done according to the circumstances recommended by the program.

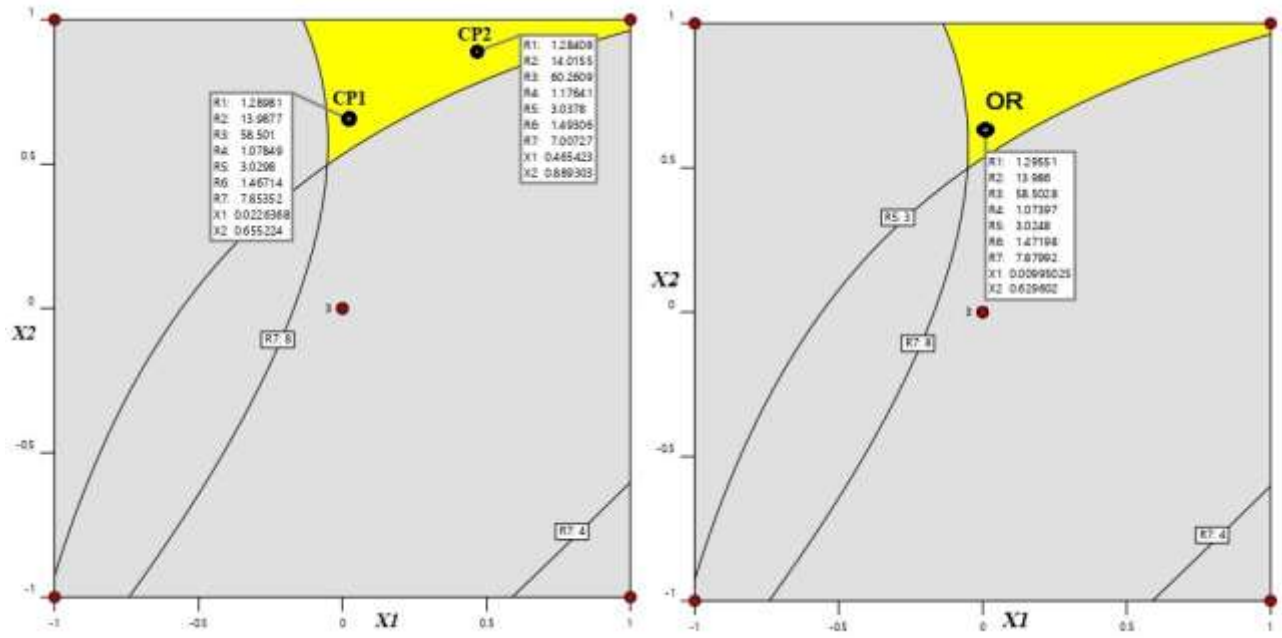

Figure 6: Selection of check point batches and outlining optimum conditions

The % PE values were calculated from the difference between experimental and predicted values. The % PE values are less than 5%, which indicate the suitability of developed models (Table 7).

Table 7: %PE values for developed mathematical model

Check point Run	Response	Experimental value	Predicted value	%PE
CP1 (Coded: X1=-0.0226, X2=0.655, X3=0) (Actual: X1=8.05, X2=2.65, X3=1)	R1	1.29	1.3	0.77
	R2	13.98	14	0.14
	R3	58.5	60.21	2.84
	R4	1.08	1.1	1.82
	R5	3.03	3	1.00
	R6	1.47	1.5	2.00
	R7	7.85	8	1.88
C2 (Coded: X1=0.465, X2=0.889, X3=0) (Actual: X1=8.93, X2=2.889, X3=1)	R1	1.28	1.3	1.54
	R2	14.02	14	0.14
	R3	60.26	61.75	2.41
	R4	1.18	1.8	34.44
	R5	3.04	3	1.33
	R6	1.49	1.5	0.66
	R7	7	7	0.00

The correlation plots of predicted value Vs experimental values were drawn for check point batches CP1 and CP2 (Figure 7). In both the plots the R2 values were found be 0.996 and 0.993 respectively for CP1 and CP2. These indicates perfect relation between predicted values and experimental value.

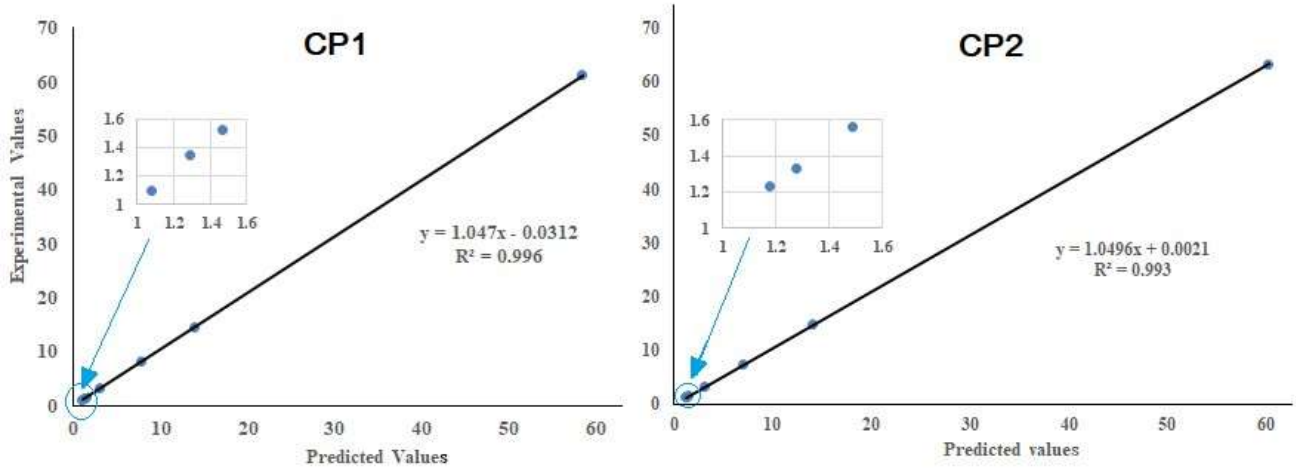

Figure 7: Correlation plots of experimental and predicted values for check point batches

4.6 Validation of developed HPLC method

The technique that was devised demonstrated specificity, since there was no interference found at the retention periods of the three medicines in the blank (diluent), confirming its specificity. The approach demonstrated good linearity throughout the tested concentrations for REM (2.5–75 µg/mL), VIL (1.25–37.5 µg/mL), and MET (25–750 µg/mL). Detailed regression equations and correlation coefficient values are presented in Table 8.

Table 8: Summary of validation parameters for REM, VIL and MET

Validation Parameter	**Acceptance criteria**	**REM**		**VIL**		**MET**	
Specificity	No interference from blank at the RT	Complies		Compiles		Complies	
Linearity (5%-150%)	Correlation Coefficient	0.998		0.998		0.998	
Range		2.5 to 75 ppm		2.5 to 75 ppm		25 to 750 ppm	
Accuracy							
Accuracy	Mean of 2 sets: 98%-102%	**% Recovery**		**% Recovery**		**% Recovery**	
50 % level		101.3		100.8		99.0	
80 % level		100.9		100.6		101.1	
100% level		99.5		99.0		99.4	
150% level		101.2		101.5		101.2	
Precision							
	Mean of 6 sets: 98%-102% %RSD: < 2.0%	**%Assay**	**%RSD**	**%Assay**	**%RSD**	**%Assay**	**%RSD**
Method Precision (n=6)		100.02	0.37	98.18	0.31	100.61	0.35

Intermediate precision		99.78	1.5	97.75	0.5	101.07	1.2
System Precision (n=5) %RSD							
%RSD of area	< 2.0 %	1.34 %		0.53%		0.51%	
Tailing factor	between 0.8 to 2.0	1.05		0.92		1.18	
Theoretical plates	NLT 1500	140615		75543		11819	
Peak purity	Purity angle should be less than purity threshold	Passed		Passed		Passed	

The technique of standard addition was used to evaluate accuracy. Remogliflozin Etabonate, Vildagliptin, and Metformin Hydrochloride standards were added to the sample solution at concentrations of 80%, 100%, 120%, and 150% of the test concentration. The test concentration corresponds to 50 µg/mL of REM, 25 µg/mL of VIL, and 500 µg/mL of MET. The concurrent recuperation of the three medications in the sample solution varied from 98% to 102%, indicating the accuracy of the procedure. The evaluation of precision covered assessing repeatability, intermediate precision, and system precision. Repeatability and intermediate accuracy of six individually designed combination solutions were assessed over many days and with various equipments. The system's accuracy was evaluated by performing six duplicate injections of a solution that was a combination of different substances. The assessment of repeatability, intermediate precision, and system precision confirmed that the %relative standard deviation (RSD) values were below 2%, satisfying the ICH criteria. The linearity plots for REM, VIL and MET are shown in Figure 8.

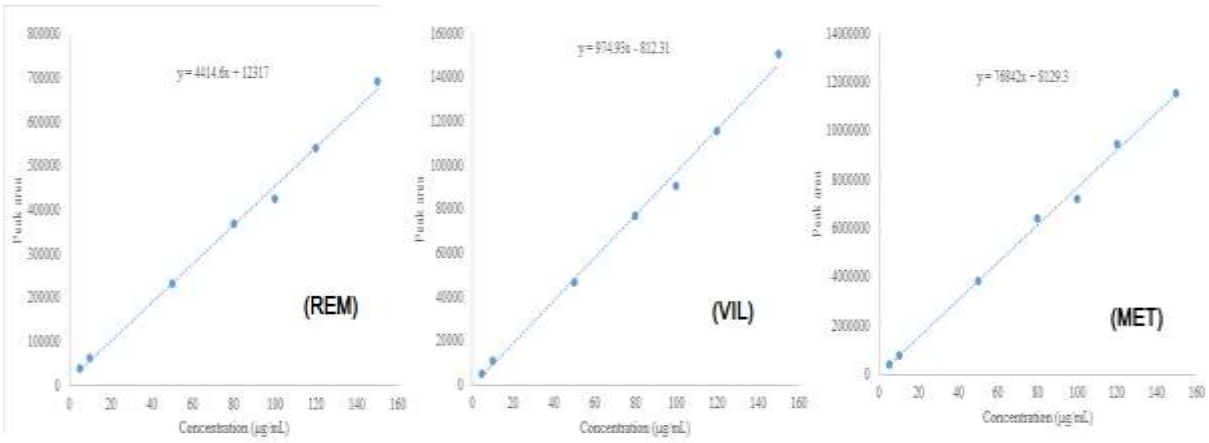

Figure 8: Linearity plots for REM, VIL and MET

5. Conclusion

Analyzing medications in fixed-dose combinations presents a notable difficulty for analysts, particularly when contrasted to dosage forms that consist of a single component. This work describes a systematic RP-HPLC technique based on AQbD for the simultaneous estimation of REM, VIL, and MET in their combination commercial tablets. In addition, the method validation was performed in compliance with the ICH standards Q2(R1). The suggested RP-HPLC technique was found to be

accurate, precise, specific, fast, and robust for separating and concurrently determining the compounds REM, VIL, and MET in mixed dose forms. The combined approach QRM and BBD was found promising in development of robust HPLC method. The developed method shows potential for ensuring the quality and conducting regular examination of the medications being studied, both in their pharmaceutical forms and in mixes generated in the laboratory. The application of this seeks to minimize the amount of waste produced in laboratories, as well as decrease the time and effort required for analysis.

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