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Risk based HPLC method development and validation for isoniazid and ethambutol using analytical QbD approach

Bhagyashri Raghunath Mogarkar*, Prakash Katakam

Department of Quality Assurance, Indira College of Pharmacy, Nanded 431606, India

Corresponding Author: Bhagyashri Raghunath Mogarkar
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ABSTRACT

Introduction: Analytical quality by design (AQbD), is a systematic approach to development that starts with established objectives and places an emphasis on understanding and control of the analytical method, based on sound science and quality risk management. The use of DoE reduces number of runs in a planned experiment and also allows the collection of higher quality data. Implementation of QbD developes the rugged and robust method which helps to comply with ICH guideline and also facilitates continuous improvement in the method.

Method: The experimental design is based on Box-behnken design of three key components of the HPLC method (mobile phase composition, flow rate and wavelength) is presented. The Zorbax Eclipse XBD-C18 (4.6mm×250mm×5µm) and mobile phase composition (90:10), flow rate (0.8 ml/min) and wavelength (212) were optimized chromatographic conditions. The approach was created according to ICH guidelines.

Result: Isoniazid and ethambutol were found to have a retention time of 4.558 min and 6.761 min respectively. The developed method had a linear range of 3-15 µg/ml and 8-40 µg/ml with a correlation coefficient of 0.9993 and 0.999 for isoniazid and ethambotol respectively. The optimized approach was accurate and precise, as evidenced by less than 2% RSD for repeatability, intraday and interday precision. The percentage recovery of spiked samples ranged from 99.45 to 100.01 as per the acceptance requirements of ICH guidelines. The validated parameters of developed method were found in the prescribed limit as per ICH guidelines.

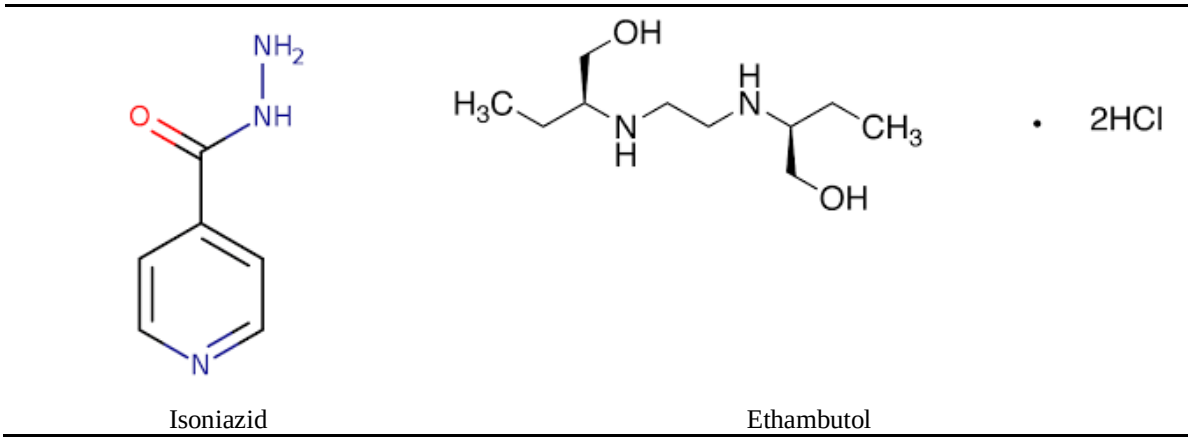
Conclusion: Using the Design Expert version 13.0.5.0, the Box-behnken experimental design explains the interaction of mobile phase composition, flow rate and wavelength at three different levels and the responses to be observed were retention time, peak area, resolution, theoretical plates and asymmetric factor. The analytical QbD approach used in method development has very much helped for better understanding of method variables hence there are less chances of failure during method validation and transfer.

Keywords: AQbD, Box-behnken, Experimental Design, DoE, Risk based analytical method development, quality risk management.

INTRODUCTION

Quality by testing (QbT), also known as trial-and-error, is a conventional approach to the development of analytical methodologies. QbT entails evaluating various operational conditions by varying one factor at a time (OFAT investigation). This unstructured method frequently necessitates a large number of trials to identify the optimal conditions. Due to an insufficient understanding of their potential impact on the method's performance, such a strategy surely does not facilitate any future modifications that may be necessary. The risk is not fully understood and cannot be managed effectively [1-6]. After identifying the working conditions, the validation phase evaluates the quality of the procedure. Therefore, quality index parameters such as precision and robustness are evaluated at the conclusion of the method development procedure. QbD is defined as ―A systematic approach to development that begins with

predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management. Equivalent to process QbD, the outcome of AQbD is well understood and fit for intended purpose with robustness throughout the lifecycle [7]. Isoniazid (ISO) also known as pyridine-4-carbohydrazide is colourless crystals or white crystalline powder. Ethambutol (ETO) is chemically 2, 2'-Ethylene(diamine)di-(2-butyl-1-ol) is a white, crystalline powder, almost colourless, and hygroscopic in nature (Fig. 1). Both are most potent and selective known tuberculostatic, anti-bacterial agent and effective in therapy of tuberculosis [8, 9].



Isoniazid Ethambutol

Fig 1: Structure of isoniazid and ethambutol

Earlier studies showed HPLC method development of isoniazid and ethambutol [10-12]. However, there is no report on HPLC method development of isoniazid and ethambutol using AQbD approach. Therefore the present investigation was performed on the risk based HPLC method development and validation for isoniazid and ethambutol using analytical QbD approach.

MATERIALS AND METHODS

Ethambutol and Isoniazid were gratis samples from Lupin Ltd., Aurangabad, India. HPLC grade methanol was obtained from Merck, Mumbai, India. Triple distilled water was prepared in house. All other chemicals were of analytical grade from Loba Chemie, Mumbai, India.

Preparation of standard stock solutions

Accurately weighted 10 mg of ISO and ETH was transferred to 10 ml calibrated volumetric flask, dissolved separately in Methanol and Water (90:10) that give final concentration of 1000 µg/ml for both the drugs.

Chromatographic conditions

On the basis of HPLC mode (Agilent 1220 LC HPLC system) and number of carbon present in molecule (analyte) stationary phase with C-18bonded phase i.e. Zorbax Eclipse XDB-C18 (4.6mm×250mm×5µ) with particle size 5 µm was selected. Isocratic mode, Mobile Phase: Methanol:Water (90:10 v/v), Injection Volume: 20 µL, UV detector at wavelength 212 nm, flow rate of 0.8 ml/min at ambient temperature.

Selection of mobile phase

The selection was made on the basis of literature survey. After assessing the solubility of both drugs in different solvents as well in mobile phases Methanol and Water in ratio 90:10 v/v was selected as a first choice.

Selection of detection wavelength

UV- detector was selected, as it is reliable and easy to set at the correct wavelength. By appropriate dilution of standard stock solution with mobile phase, varying concentrations of ETH and ISO were prepared separately. The solution were scanned using double beam UV visible spectrophotometer 1800 in the spectrum mode between the range of 400 nm to 200 nm and their overlain spectra were obtained. The analytical wavelength selected was 212 nm at which both drugs shows maximum absorbance.

Optimization of detection wavelength

Detection using UV detector at different wavelengths was performed. Physical laboratory solution containing 10 µg/ml of each ETH and ISO in the same proportion as that in Tablets was prepared in mobile phase and injected. Finally, 212nm wavelength was selected as detection wavelength.

Risk based HPLC method development by AQbD approach

HPLC method development by Analytical QbD was as follows.

Selection of quality target product profile

The QTPP is crucial for figuring out the factors that influence the QTPP parameters. For the suggested HPLC method, the retention time, peak area, resolution, theoretical plates, and peak asymmetries were identified as QTPP [13, 14].

Determine critical quality attributes

The technique parameters that have a direct impact on the QTPP are the CQAs. Three crucial technique parameters that had to be kept under control in order to keep the QTPP response range acceptable were the mobile phase composition, flow rate and wavelength. CQAs are denoted in Table 1 [15].

Factorial design

Following the establishment of the QTPP and CQAs, the mobile phase, flow rate and wavelength of the HPLC technique were optimised and chosen using the Box-behnken experimental design. Using a Box-behnken statistical screening strategy, the different interactions and quadratic effects of the mobile phase composition, flow rate and wavelength on the retention time, theoretical plates, resolution, peak area and peak asymmetry were investigated. The optimum answer for a second-order polynomial exploring quadratic response surfaces was a 3-factor, mobile phase composition, flow rate and wavelength at 3 distinct levels, design with Design Expert® Trial Version 13.0.5.0 [14].

$$Y = \beta_O+\beta_1A+\beta_2A+\beta_3A+\beta_4AB+\beta_5AC+\beta_6BC+\beta_7A^2+\beta_8B^2+\beta_9C^2$$

When Y is the measured response connected to each combination of factor level, A, B and C are independent variables coded for levels, β_O is an intercept (value of Y when X = 0), and β_1 to β_9 are regression coefficients generated from experimental runs of the recorded experimental values of Y. The factors were chosen based on preliminary analysis because multivariable interactions of variables and process parameters have been examined [16]. Table 1 lists the mobile phase composition, flow rate and wavelength that were selected as independent variables. Retention time, peak area, resolution, theoretical plate and peak asymmetry served as the dependent variables for the suggested independent variables [17].

Evaluation of experimental results and selection of final method condition

The Box-behnken method was used to evaluate these method conditions. The initial step involved evaluating the peak area, resolution, peak asymmetries, theoretical plates, and retention time conditions. This led to distinctive chromatographic conditions for ethambutol and isoniazid. The demonstrated acceptable ranges are from stable areas where purposeful changes to the procedure parameters have no impact on the quality.

This guarantees that the method won't fail later on when being validated. The variable must be optimised at various levels until the responses were within the acceptable ranges if the modelling tests do not get the intended response [18]. Utilising the Design Expert tools, the ideal chromatographic conditions for the suggested independent variables must be determined.

Risk assessment

The created method's attributes, such as its efficiency and ability to function throughout the duration of the product's life, are taken into consideration when choosing the optimised final method. A risk-based strategy built on the evaluation of the approach to evaluate the robustness and ruggedness was conducted using the AQbD principles outlined in the ICH Q8 and ICH Q9 guidelines [19]. For robustness and ruggedness investigations, the parameters of the method or its performance under multiple conditions, such as different chemicals, analysts, instruments, reagents, pH, wavelength, and days, were assessed [20].

Implementation of control strategy

The implementation of a control strategy should come after the development of the method. For the creation of the analytical control plan, the analytical target profile was established. The planned set of controls known as the analytical control strategy was formed from an awareness of the many parameters, including risk management, analytical method, and suitability for purpose. All of these variables guarantee that the method's effectiveness and the outputs' quality fall within the intended analytical target profile. Planned analytical control procedures included sample preparation, measurement, and replication control procedures [21].

Continual improvement for managing analytical life cycle

The best method for managing the analysis lifecycle is to continuously improve, which can be done in the laboratory by keeping an eye on the consistency of the quality and performing routine maintenance on HPLC equipment, computers, and software [22].

Table 1: Factors identified as critical quality attributes.

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Composition	90.00	70.00	90.00	0.0000	Actual
B	Flowrate	0.8000	0.7000	0.9000	0.0000	Actual
C	Wavelength	212.00	208.00	212.00	0.0000	Actual

Analytical method validation

Analytical method validation is a documentary evidence that provides a high degree of assurance for a specific method which denotes the process used to confirm the analytical process is suitable for its intended use. The developed HPLC method for estimation of ethambutol and isoniazid was validated as per ICH Q2 (R1) guidelines [23].

Recovery study

To the pre-analysed sample solutions (10 µg/ml of ETH and 10 µg/ml of ISO), a known amount of standard solutions of the pure drugs (ETH and ISO) were added i.e. 16, 24, and 40 µg/m1 of ETH and 9, 12 and 15 µg/ml of ISO (standard stock solution) was added, and total conc. of above dilution is measured by using equation I and II.

$$\% \text{ Recovery} = A / (B+C)$$

Where,
A = Total amount of drug estimated, B = Amount of drug found on reanalyzed bases and C = Amount of pure drug added

Validation parameters

Appropriate aliquots of the standard stock solutions of ETH and ISO were pipetted out and transferred to a series of 10 ml calibrated volumetric flasks respectively. The volume was made up to the mark with mobile phase to obtain working standard solutions of ETH of concentrations 8, 16, 24, 32, and 40µg/ml and ISOof concentrations 3, 6, 9, 12 and 15µg/ml. From these solutions, 20 µl injections of each concentration of the drug were injected into the HPLC system three times separately and chromatographed under the conditions as described above. Evaluation of the drug was performed with the UV detector set at 212 nm and the peak areas were recorded. The standard calibration curve for ETH and ISO was plotted separately as peak area Vs the respective concentration of ETH and ISO.

Precision of an analytical method is the degree of agreement among individual test results. It is expressed as ± S.D. or % RSD of series of measurements. Precision of the method was verified by using stock solutions in the ratio of 24:9 containing 24 µg/ml ETH and 9 µg/ml of ISO. System repeatability was done by repeating the assay three times of three replicate dilutions of the same concentration after every two hours on the same day for intraday precision. Interday precision was carried out by performing the assay of three sample sets after 24 and 48 h.

Accuracy is measured by using 3 concentration levels of 8, 24 and 40 ug/ml for ETH and 3, 9 and 15 ug/ml for ISO, by making triplicate preparations at each level. Robustness expresses the precision within laboratories, Variation like different wavelength and pH. Robustness of the methods was assessed by carrying out assay 3 times with different wavelength and pH by using same equipment.

Ruggedness of the proposed method is determined by using linearity parameter. Working standard solutions of ETH of concentrations 8, 16, 24, 32, and 40µg/ml and ISO of concentrations 3, 6, 9, 12 and 15µg/ml made and injected 20 µl of each concentration of the drug into the HPLC system three times separately and chromatographed under the conditions as described above. Evaluation of the drug was performed with the UV detector set at 212 nm and the peak areas were recorded. The standard calibration curve for ETH and ISO was plotted separately as peak area vs the respective concentration of ETH and ISO.

The detection limit of an individual analytical procedure is the lowest amount of an analyte in a sample, which can be detected but not necessarily quantitated as an exact value. The limit of detection (LOD) may be expressed as,

$$\mathbf{LOQ} = \frac{\mathbf{3.3 \times Std.Deviation}(\sigma)}{\mathbf{Slope}\ (\mathbf{S})}$$

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The limit of quantitation (LOQ) may be expressed as

$$\mathbf{LOQ} = \frac{\mathbf{10 \times Std.Deviation}(\sigma)}{\mathbf{Slope}\ (\mathbf{S})}$$

System suitability is a Pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from five replicate injection of standard drug solution.

Assay

Twenty tablets were weighed and powdered. Powder (19.2681mg) equivalent to 10 mg of both ETH and ISO weighed accurately, transferred to 10 ml of volumetric flask and add 10 ml of solvent to get stock solution of 1000 µg/ml. Further dilution prepared to get concentration of ETH of 24 µg/ml and ISO of 9 µg/ml. The solution was analysed by HPLC with same chromatographic condition as linearity and assay is determined.

Degradation study

Forced degradation is also called as stress testing, is carried out to demonstrate as specificity to developed a stability-indicating analytical method, using high-performance liquid chromatography (HPLC). Forced degradations were carried out under thermolytic, photolytic, acid, base and oxidative stress conditions.

RESULTS AND DISCUSSION

HPLC method development by AQbD approach

Selection of quality target product profile

Retention time, peak area, resolution, theoretical plates, and peak asymmetries were the QTPP chosen for the improvement of HPLC chromatographic conditions [24].

Determine critical quality attributes

The mobile phase combination methanol to water, 90:10, flow rate 0.8 ml/min and wavelength 212 were identified as critical quality attributes.

Factorial design

The proposed HPLC method development was chosen to utilise the Box-Behnken design. Table 2 displays the optimisation of several parameters.

Design space

A total of 17 runs of the quadric design model with the Box-Behnken design and response surface research type were employed.

The proposed Box-Behnken experimental design was used, and the results were summarised after the mobile phase composition, flow rate and wavelength were assessed in relation to the nine responses of ISO retention time, ETH retention time, ISO area, ETH area, resolution, ISO theoretical plates, ETH theoretical plates, ISO peak symmetry and ETH peak asymmetry. Table 2 shows the optimization of parameters for analysis of ETH and ISO using Box-Behnken design.

Table 2: Optimization of parameters for analysis of ETH and ISO using Box-Behnken design

Run	Composition %	Flowrate Ml/min	Wave length nm	ISO RT min	ETH RT min	ISO Area units	ETH Area units	Reso lution units	ISO TP units	ETH TP units	ISO AF units	ETH AF units
1	80	0.9	212	5.218	8.553	289415	667884	5.64	6401	7170	1.35	1.24
2	70	0.8	212	9.399	14.878	221801	791262	5.16	3317	7171	1.29	1.07
3	80	0.8	210	5.765	9.468	223177	741339	5.84	6422	8535	1.36	1.26
4	90	0.9	210	4.083	6.012	226699	582903	4.99	7776	8159	1.36	1.28
5	80	0.7	208	6.553	10.741	234377	836640	6.36	6966	8084	1.32	1.25
6	90	0.8	208	4.558	6.753	296331	726081	4.86	7167	8182	1.32	1.23
7	70	0.8	208	9.415	14.917	307964	757437	5.45	7505	8433	1.22	1.02
8	80	0.8	210	5.765	9.468	223177	741339	5.84	6422	8535	1.36	1.26
9	70	0.9	210	8.55	13.412	228821	682484	4.87	5271	7851	1.21	1.1
10	80	0.8	210	5.765	9.468	223177	741339	5.84	6422	8535	1.36	1.26
11	80	0.9	208	5.175	8.521	204282	665158	5.93	6524	8436	1.31	1.26
12	80	0.8	210	5.765	9.468	223177	741339	5.84	6422	8535	1.36	1.26
13	90	0.8	212	4.558	6.761	313825	702455	4.81	7232	8076	1.34	1.28
14	70	0.7	210	10.602	16.844	235539	861408	6.52	7029	8083	1.29	1.24
15	80	0.7	212	6.571	10.791	266723	850523	6.2	6497	8257	1.33	1.28
16	90	0.7	210	4.883	7.388	245605	855422	4.77	5645	7863	1.38	1.28
17	80	0.8	210	5.765	9.468	223177	741339	5.84	6422	8535	1.36	1.26

Design Analysis

The obtained equations for response surface quadratic model were as follows:

Response 1: Retention time of isoniazid

From fig. 2 and equation ISO retention time (for actual values)=510.927–2.43515×A–51.48125×B–3.56881×C+0.313×AB+0.0002×AC+0.03125×BC+0.011839×A^2+8.0625×B^2+0.008406×C^2, it was concluded that as β1 negative coefficient (–2.43515) suggests that as the amount of methanol in the mobile phase (A) decreases, β2 negative coefficient (–51.48125) suggests that as flow rate decreases and β3 negative coefficient (–3.56881) suggests that as wavelength decreases, the value of retention time of isoniazid was increased.

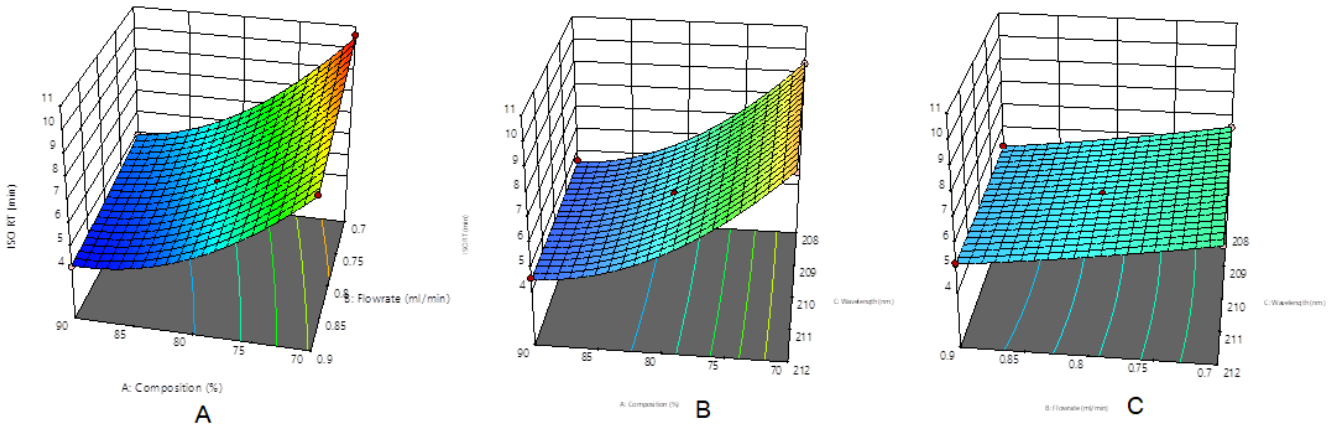


Fig 2: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R1 retention time of ISO

Response 2: Retention time of ethambutol

From fig. 3 and equation ETH retention time (for actual values) = 716.066–3.04619×A–69.5975×B–5.10519×C+0.514×AB+0.000588×AC–0.0225×BC+0.013109×A^2+13.5125×B^2+0.012094×C^2, it was concluded that as β1

negative coefficient (–3.04619) suggests that as the amount of methanol in the mobile phase (A) decreases, β2 negative coefficient (–69.5975) suggests that as flow rate decreases and β3 negative coefficient (–5.10519) suggests that as wavelength decreases, the value of retention time of ethambutol was increased.

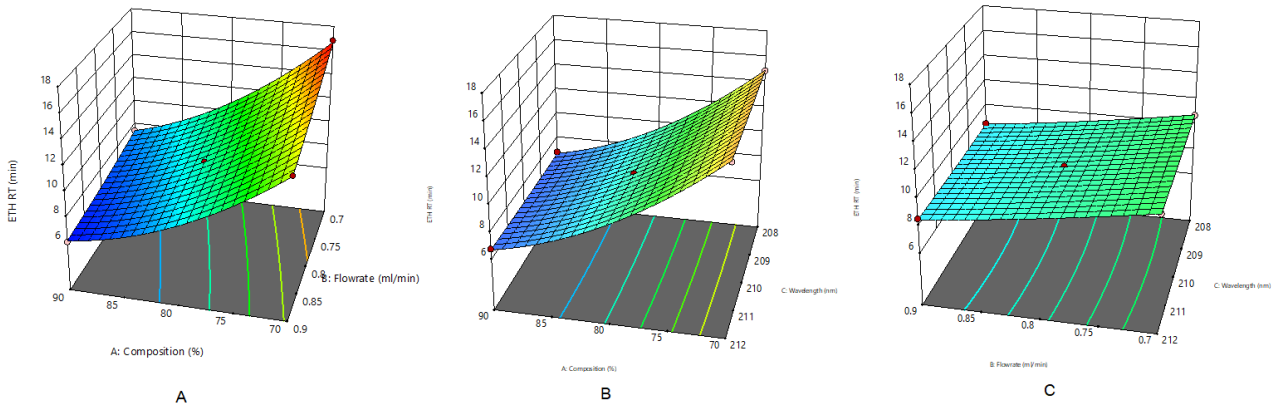


Fig 3: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R2 retention time of ETH

Response 3: Peak area of isoniazid

From fig. 4 and equation ISO peak area (for actual values) = 453694000–306373.83×A–11630751.24×B–4161059.62501×C–3047×AB+1295.71×AC+65983.75×BC+236.34×A^2–1264600×B^2+9542.06×C^2, it was concluded that as β1 negative coefficient (–306373.83) suggests that as the amount of methanol in the mobile phase (A) decreases, β2 negative coefficient (–11630751.24) suggests that as flow rate decreases and β3 negative coefficient (–4161059.62501) suggests that as wavelength decreases, the value of peak area of isoniazid was increased.

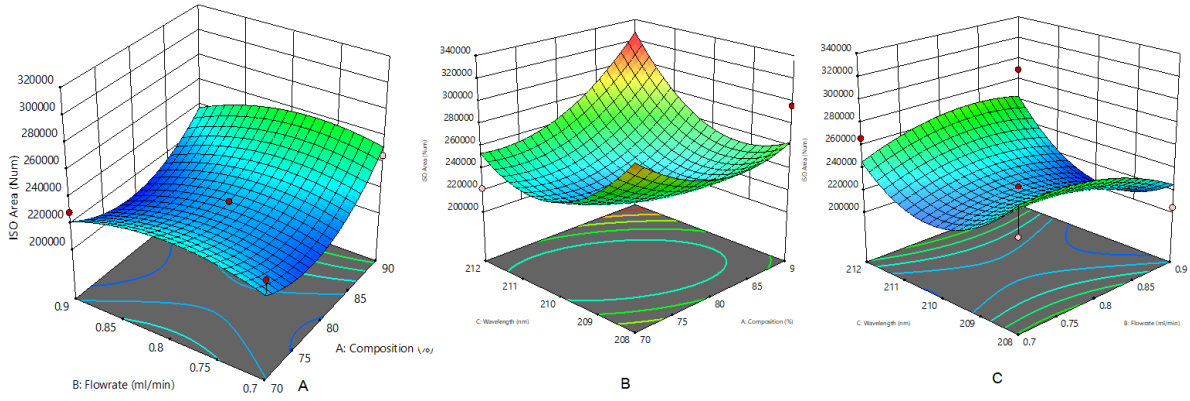


Fig 4: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R3 peak area of ISO

Response 4: Peak area of ethambutol

From fig. 5 and equation ETH peak area (for actual values) = 54508300+171928×A+2597040×B–584221×C–23398.8×AB–718.138×AC–13946.3×BC–32.6363×A^2+747887×B^2+1558.344×C^2, it was concluded that as β1 positive coefficient (171928) suggests that as the amount of methanol in the mobile phase (A) increases, β2 positive coefficient (2597040) suggests that as flow rate increases and β3 negative coefficient (–584221) suggests that as wavelength decreases, the value of peak area of ethambutol was increased.

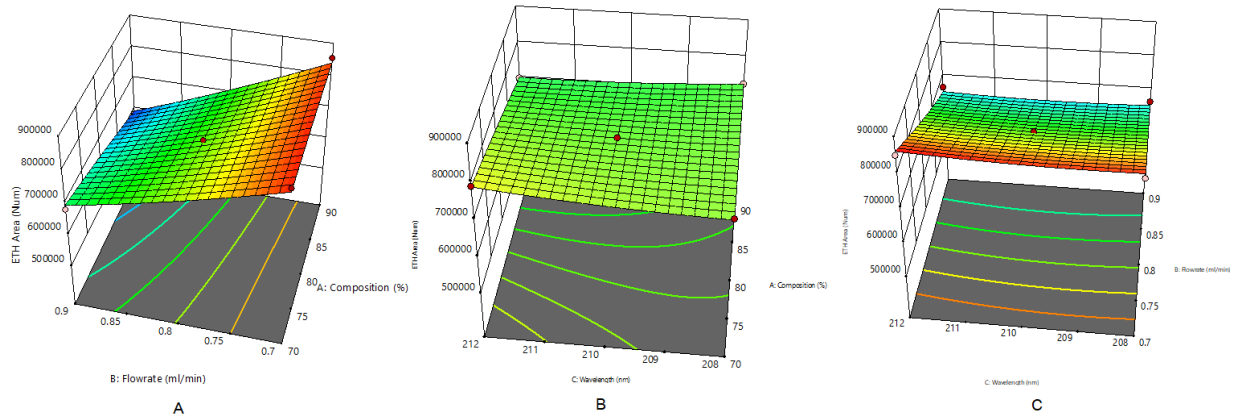


Fig 5: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R4 peak area of ETH

Response 5: resolution of isoniazid and ethambutol

From Fig. 6 and equation resolution (for actual values) = –98.95375+0.175875×A–39.1×B+1.15312×C+0.4675×AB+0.003×AC–0.1625×BC–0.007575×A^2+20.5×B^2–0.003125×C^2, it was concluded that as β1 positive coefficient (0.175875) suggests that as the amount of methanol in the mobile phase (A) increases, β2 negative coefficient (–39.1) suggests that as flow rate decreases and β3 positive coefficient (1.15312) suggests that as wavelength increases, the value of resolution of isoniazid and ethambutol was increased.

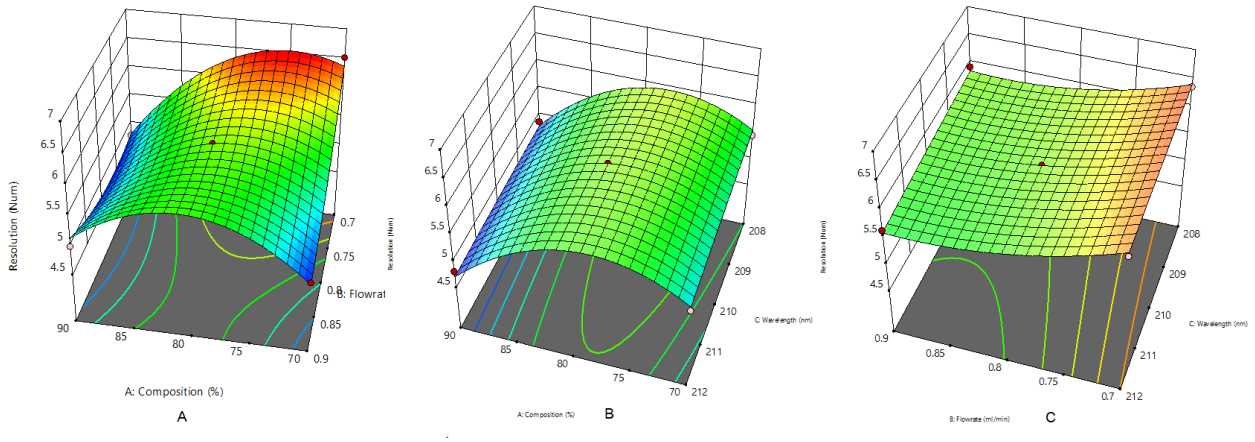


Fig 6: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R5 resolution of ISO AND ETH

Response 6: Theoretical plates of isoniazid

From fig. 7 and equation ISO theoretical plates (for actual values) = 1367940–11656.4×A–192811×B–7518.69×C+972.25×AB+53.1625×AC+432.5×BC–1.4175×A^2+15000×B^2+6.25×C^2, it was concluded that as β1 negative coefficient (–11656.4) suggests that as the amount of methanol in the mobile phase (A) decreases, β2 negative coefficient (–192811) suggests that as flow rate decreases and β3 negative coefficient (–7518.69) suggests that as wavelength decreases, the value of theoretical platesof isoniazid was increased.

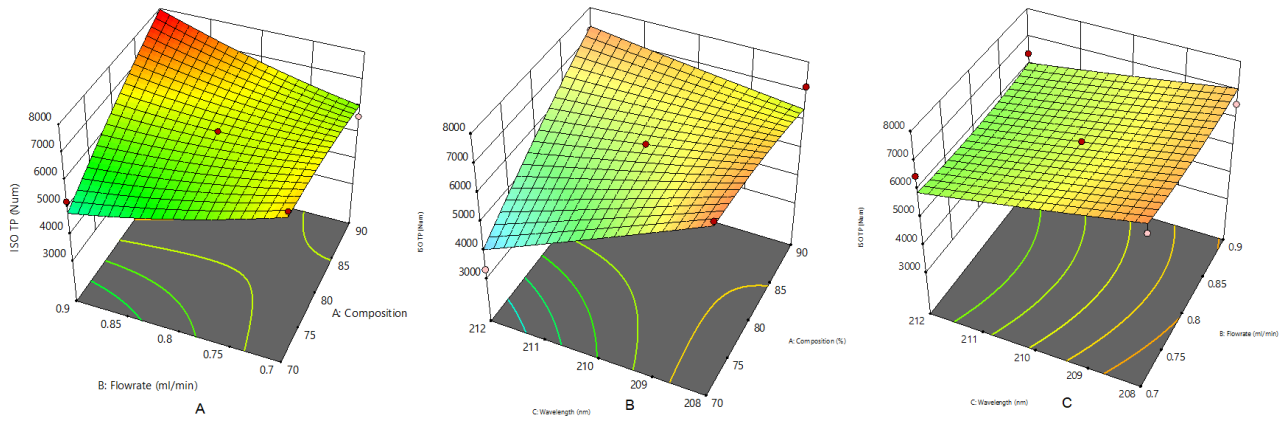


Fig 7: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R6 theoretical plates of ISO

Response 7: Theoretical plates of ethambutol

From fig. 8 and equation ETH theoretical plates (for actual values) = –3196930–2677.03×A+408319×B+30146.06×C+132×AB+14.45×AC–1798.75×BC–2.83625×A^2–26237.5×B^2–71.4688×C^2, it was concluded that as β1 negative coefficient (–2677.03) suggests that as the amount of methanol in the mobile phase (A) decreases, β2 positive coefficient (408319) suggests that as flow rate increases and β3 positive coefficient (30146.06) suggests that as wavelength increases, the value of theoretical platesof ethambutol was increased.

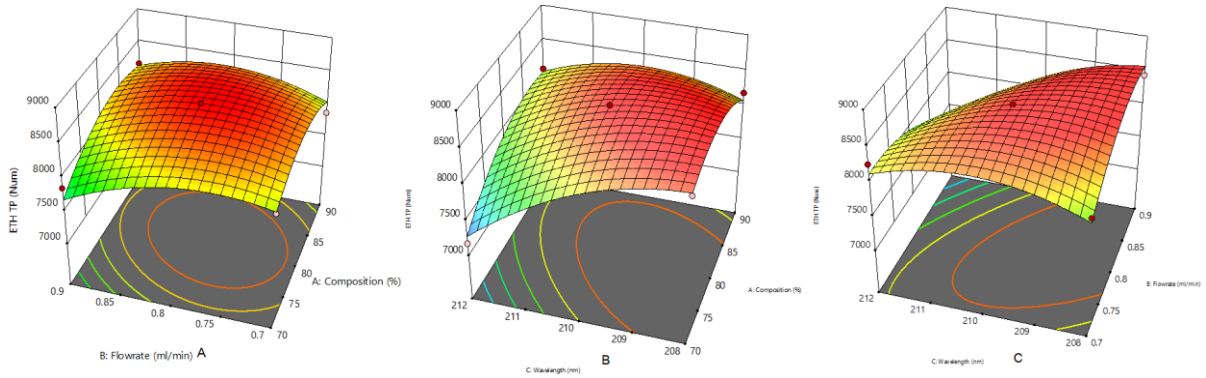


Fig 8: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R7 theoretical plates of ETH

Response 8: Assymetry factor of isoniazid

From fig. 9 and equation ISO asymmetry factor (for actual values) = –282.843+0.192125×A–7.9875×B+2.65375×C+0.015×AB–0.00063×AC+0.0375×BC–0.00043×A^2–0.75×B^2–0.00625×C^2, it was concluded that as β1 positive coefficient (0.192125) suggests that as the amount of methanol in the mobile phase (A) increases, β2 negative coefficient (–7.9875) suggests that as flow rate decreases and β3 positive coefficient (2.65375) suggests that as wavelength increases, the value of peak asymmetry of isoniazid was increased.

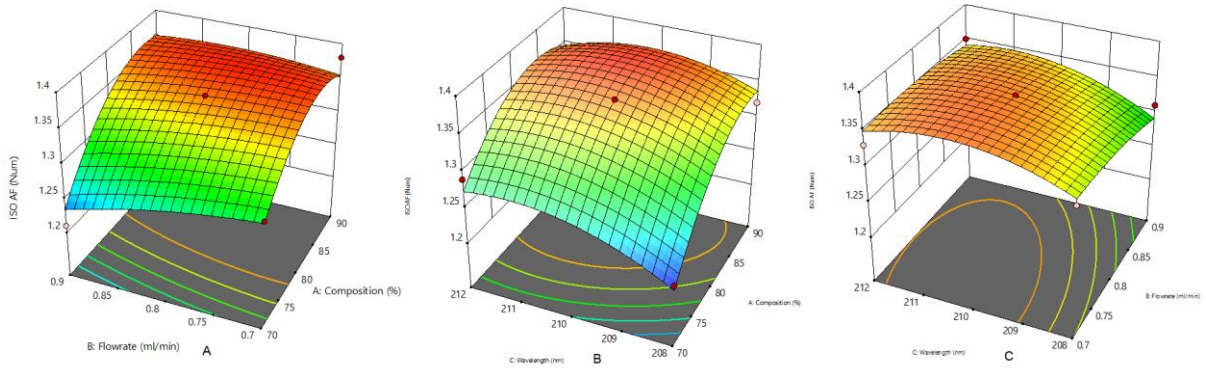


Fig 9: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R8 asymmetry factor of ISO

Response 9: Asymmetry factor of ethambutol

From fig. 10 and equation ETH asymmetry factor (for actual values) = –438.3725+0.094×A+4.3125×B+4.12562×C+0.035×AB+ 0.00000000000000062×AC–0.0625×BC–0.000712×A^2+3.625×B^2–0.009687×C^2, it was concluded that as β1 positive coefficient (0.094) suggests that as the amount of methanol in the mobile phase (A) increases, β2 positive coefficient (4.3125) suggests that as flow rate increases and β3 positive coefficient (4.12562) suggests that as wavelength increases, the value of peak asymmetry of ethambutol was increased.

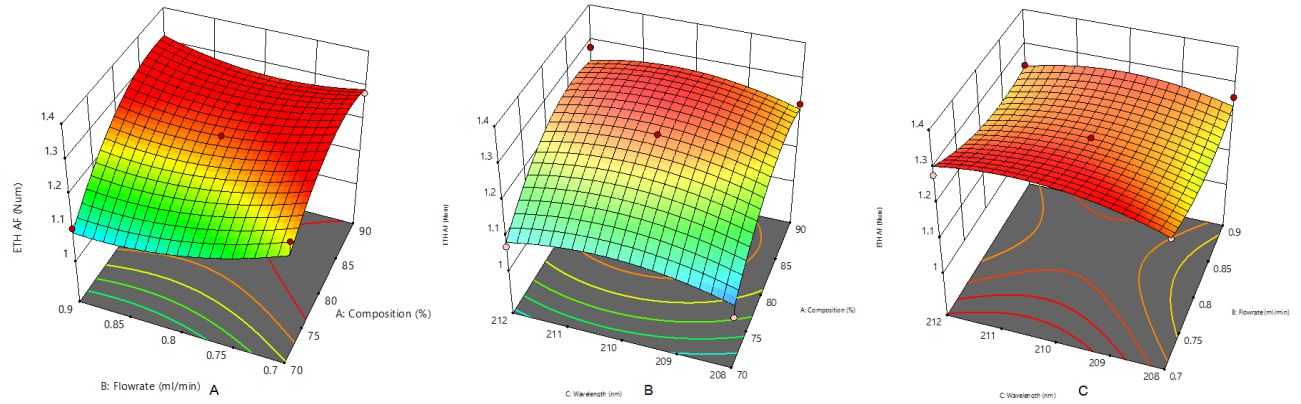


Fig. 10: 3D surface plot for effective combination of factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength on R9 asymmetry factor of ETH

The obtained solution for optimized formulation and their correlation values are shown in Table 3.

Table 3: Obtained solution for optimized formulation and their correlation values

Code	Compo –sition %	Flow rate Ml/min	Wave length nm	ISO RT min	ETH RT min	ISO Area units	ETH Area units	Reso–lution units	ISO TP units	ETH TP units	ISO AF units	ETH AF units	Desir–ability
C18	88.922	0.830	212.000	4.417	6.684	320003.448	673991.901	4.935	7508.402	7936.133	1.353	1.252	0.335
R^2	-	-	-	0.9997	0.9998	0.8585	0.9934	0.9917	0.9237	0.9784	0.9376	0.9625	

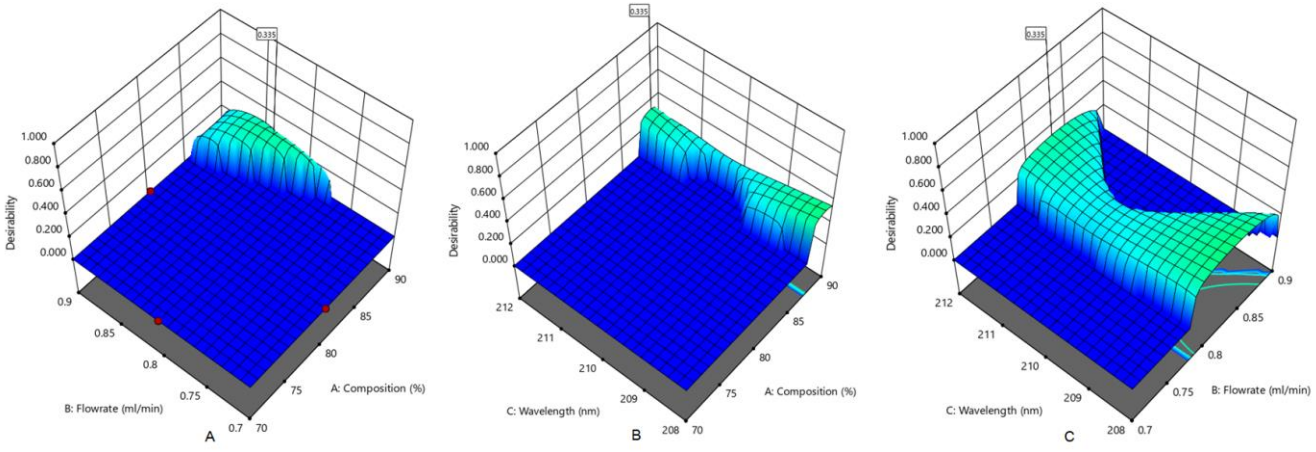


Fig. 11: 3D surface plot of desirability for factors (A) A-Composition and B-Flow rate, (B) A-Composition and C-Wavelength and (C) B-Flow Rate and C-Wavelength to obtain optimized formulation

Confirmation

The data mean of all analytical target product profiles are in between lower and upper limit hence the confidence interval for analytical target product profile of validated HPLC method is two–sided. Confidence level for analytical target product profile of validated HPLC method is 95%, hence 95% of times we expect to reproduce the estimate between lower and upper limit of the confidence interval.

Validation parameters

Good linearity was obtained in the concentration range of 8-40 µg/ml and 3-15 µg/ml of ETH and ISO respectively.The R^2 value is determined near to one (Figs. 12 and 13). The standard working curve equation for ETH was found to be Y = 50645x - 10669 with a correlation coefficient value of r^2 = 0.999. The standard working curve equation for ISO was found to be Y = 44356x+61374with a correlation coefficient value ofr^2 = 0.999.

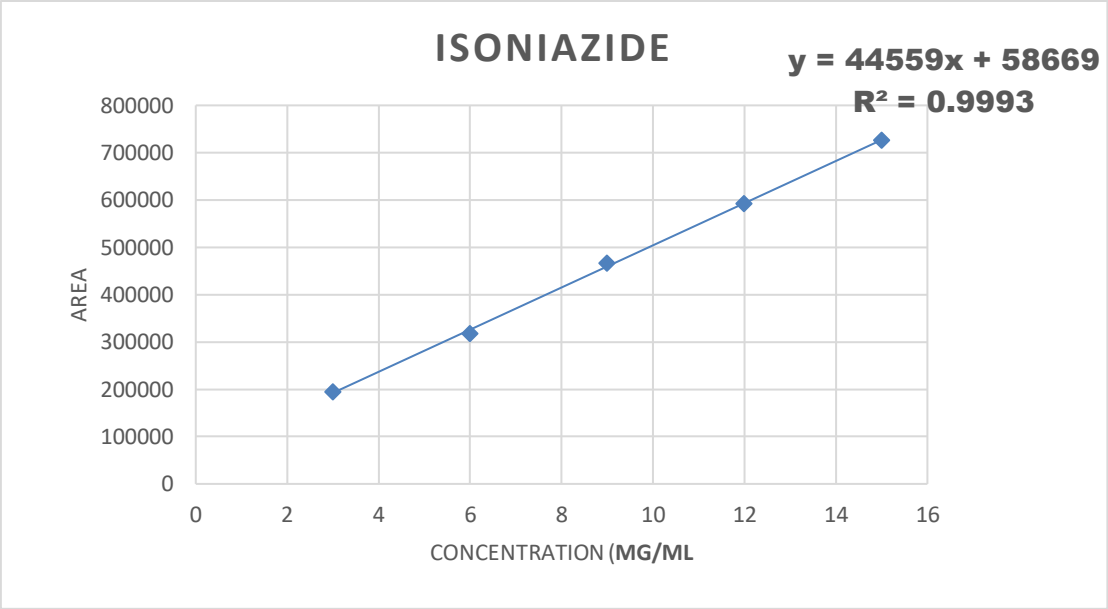


Fig 12 : calibration curve of ISO

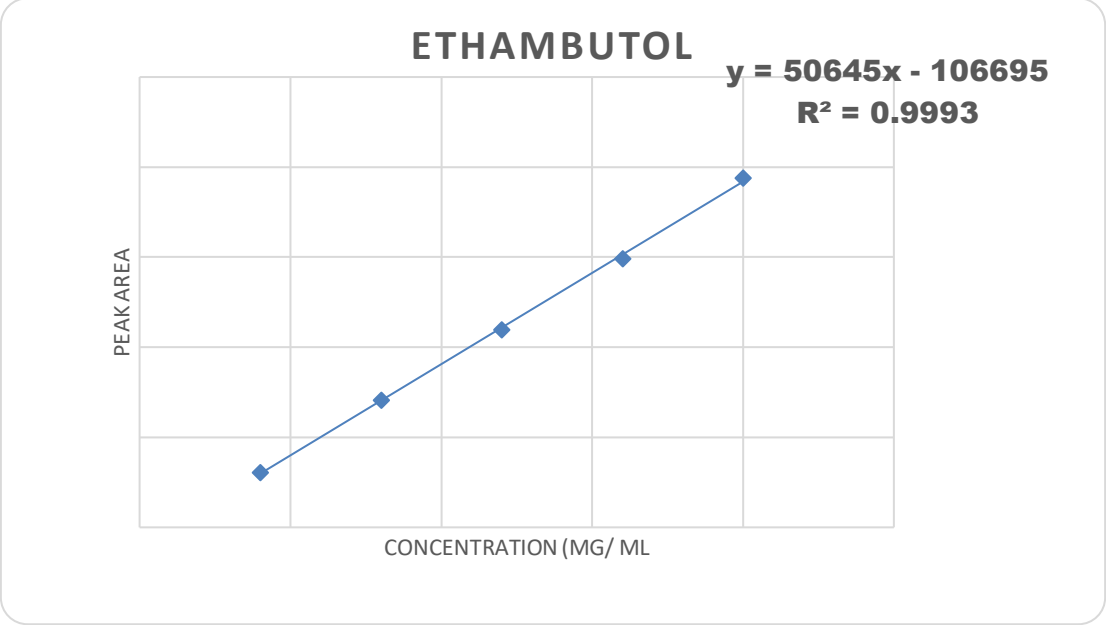


Fig 13: calibration curve of ETH

Interday precision was carried out by performing the assay of three sample sets after 24 hours and 48 hours. The %RSD for interday precision was found to be 0.211 and 0.0528 for ISO and ETH. The %RSD for intraday precision was found to be 0.027 and 0.097 for ISO and ETH. The results of accuracy showed the %RSD for interday precision was found to be 0.291 and 0.096 for ISO and ETH. The %RSD values for robustness was found to be 0.454 and 0.429 for ISO and TH. The results of pH stability at pH 2.8, 3.0 and 3.2 showed %RSD of 0.398 and 0.078 for ISO and ETH. The obtained %RSD values are less than 2 which are within the limits. The R2 values for ruggedness were found to be 0.9993 and 0.999 for ISO and ISO. The LOD of ISO was found 0.110 and for ETH it was 0.070. Similarly the LOQ of ISO and ETH were found 0.334 and 0.210 respectively. The summary of validation parameters for Isoniazid and Ethambutol are reported in Table 4.

Table 4: Summary of Validation Parameter of ISO and ETH

Parameters	ISO	ETH
Linearity range [µg/ml]	3–15	8–40
Regression equation [Y = mX+C]	y = 44356x+61374	y = 50645x–106695
Recovery [%]	99.45 to 99.49	99.75 to 100.01
Precision [% RSD]		
Intra–day [n = 3]	0.052848339	0.096821435
Inter–day [n = 3]	0.211331357	0.027210652
Accuracy [% RSD]	0.291268924	0.096399126
Robustness [% SD]		
For different wavelength	0.4544851	0.4293445
For different pH	0.3986427	0.0788281
Ruggedness [R^2]	0.9993	0.999
LOD [µg/ml]	0.1103	0.07
LOQ [µg/ml]	0.33425	0.21
Assay [%]	99.544778	99.898978
Degradation [%]	1–12	0–14

The assay values were found 99.54% and 99.89% respectively for ISO and ISO. The degradation studies are summarized in Table 5.

Table 5: Result of degradation study for ISO and ETH

Degradation	ISO (%)	ETH (%)
Acid Degradation	8.618228029	10.23578682
Base Degradation	11.68643066	13.89296318
H2O2 Degradation	5.030145074	3.213649639
Photolytic Degradation	1.392634937	0.193814325
Thermal Degradation	2.719596432	1.079064601

DISCUSSION

The analytical quality-by-design HPLC method has been devised to estimate the amount of isoniazid and ethambutol in pharmaceutical formulations. For the HPLC analysis of isoniazid and ethambutol, the analytical target product profile included retention time, peak area, resolution, theoretical plates, and peak asymmetry. The three factors, mobile phase composition, flow rate and wavelength were shown as the critical quality attributes that have the greatest impact on the analytical target product profile. Using Design Expert Software Trial Version 13.0, the Box-Behnken design was applied to three factors at three separate levels. The crucial factors that affect the analytical target profile were discovered by the risk assessment study [25, 27]. When performing chromatographic separation, the variability of the column used, the instrument set up, and the injection volume were all maintained under control, while factors like the pH of the mobile phase, the flow rate, wavelength and the column temperature were given to a robustness study.

The HPLC method for isoniazid and ethambutol was successfully developed using the analytical quality-by-design methodology. The Zorbax Eclipse XBD–C18 column (250×4.6 mm, 5 µm particle size) and mobile phase consist of methanol to water, 90:10 v/v, flow rate 0.8 ml/min and wavelength 212 nm were used in the optimised RP–HPLC technique of isoniazid and ethambutol. Isoniazid and ethambutol was found to have a retention time of 4.558 min and 6.761 min respectively. The method had a linear range of 3–15µg/ml and 8–40 µg/ml with a correlation coefficient of 0.9993 and 0.999 for isoniazid and ethambutol respectively. The optimised approach was accurate and precise, as evidenced by the less than 2% RSD for repeatability, intraday, and interday precision. The LOD and LOQ were 0.1103 µg/ml, 0.07 µg/ml and 0.33425 µg/ml, 0.21 µg/ml for isoniazid, ethambutol respectively. The percentage recovery of spiked samples ranged from 99.45 to 100.01 as per the acceptance requirements of ICH guidelines. The approach was created in accordance with ICH guidelines.

CONCLUSION

The analytical quality-by-design HPLC method has been devised to estimate the amount of isoniazid and ethambutol in pharmaceutical formulations. For the HPLC analysis of isoniazid and ethambutol, the analytical target product profile included retention time, peak area, resolution, theoretical plates, and peak asymmetry. The crucial factors that affect the analytical target profile were discovered by the risk assessment study. The HPLC method for isoniazid and ethambutol was successfully developed using the analytical quality-by-design methodology. This method could help the industry to implement the technique of AQbD for simultaneous estimation of isoniazid and ethambutol using RP-HPLC.

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