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Development and validation of QbD approach in RP-HPLC analytical method for separation and identification of anti-tuberculosis drug Rifapentine

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ABSTRACT

Background: Tuberculosis (TB) poses both a global health and economic burden. Timely treatment is essential but costly and slow. HPLC, enhanced by QbD, ensures precise drug analysis, improving efficiency and reliability in TB treatment.

Objective: The present study aims to develop the QbD approach RP-HPLC method for the separation and identification of the anti-tuberculin drug rifapentine.

Methodology: The current study focused on optimising RT-HPLC conditions, including the mobile phase, flow rate, and wavelength, for the analysis of rifapentine. 20 µg of rifapentine was prepared using HPLC-grade methanol. The chromatographic conditions were optimised with BBD Design Expert Software v13. Analysis was performed using a C18 reversed-phase column (250 mm x 4.6 mm, 5.0 µm) with a diode array detector (DAD). The mobile phase was a mixture of methanol and 1% orthophosphate acid (81:19, v/v), with a flow rate of 1.1 mL and an injection volume of 20 µL. The validation and data analysis were conducted at room temperature with a retention time of 5 minutes at 252 nm.

Results: The developed method demonstrated linearity with r^2=0.1705 and statistical significance ($p<0.0001$). The chromatography peal purity confirmed the absence of coeluting peaks with the rifapentine peak. The system suitability parameters, including the tailing factor and theoretical plate, were 1.15 and 7965, respectively. Additionally. The method's validation factors were within the limits specified by ICH guidelines.

Conclusion: The QbD approach for developing the rifapentine analytical method proved accurate and specific, meeting ICH regulatory norms. RP-HPLC by QbD is a reliable method that ensures validity and high-quality drug production, supports effective TB treatment, and facilitates quality checks and bulk production of the pharmaceutical formulation.

Key words: RP-HPLC, tuberculosis, QbD analytical method, rifapentine, pharmaceutical formulation, high-quality drug

INTRODUCTION

The safety, efficacy, and quality of drugs are crucial in therapeutic analysis.(1–3) One major advancement in the therapeutics field is the implementation of the Quality by Design (QbD) approach, which integrates quality into drug development from the outset. QbD emphases on understanding and controlling variability in the manufacturing process, shifting from traditional quality control to a more practical approach that enhances product consistency and aligns with regulatory expectations.(4–9)

QbD is particularly effective in developing and validating analytical methods, including liquid chromatography techniques like HPLC, RP-HPLC, LC-MS, and size exclusion chromatography.(10–13) These methods are essential for identifying and separating therapeutic compounds. RP-HPLC, in particular, is versatile and widely used in the analysis of various drugs, such as anti-tuberculosis agents like rifapentine, isoniazid, pyrazinamide, and ethambutol.(14–16) Rifapentine is critical in TB treatment, and given the global rise of TB, a reliable analytical method is essential for its identification and separation in bulk production.

Our research focuses on developing an RP-HPLC method for rifapentine using the QbD approach, ensuring quality and potency in pharmaceutical formulations. The systematic QbD process involves defining the analytical target profile, identifying critical quality attributes, and conducting risk assessments.(4,6,9,13) By understanding the influence of critical parameters, such as mobile phase composition and flow rate, we optimise method performance. Validation steps, in line with ICH guidelines,

confirm the method's reliability, ensuring accuracy, precision, and robustness.

MATERIALS AND METHOD

The university ethical committee at Bhopal Nobel's University in Udaipur, India, ethically approved the study. We performed seven trails for standardisation, achieving it in the eighth and final trail, after which we initiated method validation.

1. Analytical setup and chromatographic parameter

RP-HPLC analysis of rifapentine was performed with Agilent Tech. Gradient System 1100 with an auto injector and quaternary gradient pump (Mod. No.: G130A) with CHEMSTATION 10.1 software. The DAD-UV detector (Mod. No.: G13148) was used for the sample component detection. The C18 (Fortis) reverse-phase column of size 250mm x 4.6mm with a 5.0µm particle size was used for the separation of the sample components. The temperature was set at room temperature (27 °C to 28 °C). The chromatographic conditions were set with a C18 reverse-phase column equilibrated with a mobile phase comprising methanol and 1% orthophosphoric acid (81:19, v/v) with a diode array detector. The mobile phase pH was adjusted to 6 with a flow rate of 1.1 mL/min and a wavelength of 252 nm, and the injection volume was 20 µL. The method optimisation was done for various parameters, such as mobile phase, flow rate, and wavelength, with four response factors. All chemicals and reagents used were of analytical grade. The solvents, 1% orthophosphoric acid and methanol, were of HPLC grade.

2. Preparation of standard, stock solution, quality control sample, and wavelength selection

The 10 mg of rifapentine was used as a standard procured from Megafine Pharma Pvt. Ltd., Nasik, India. 10 mg of rifapentine was diluted in 10 mL of 0.1% OPA and methanol in the mobile phase with a final concentration of 1000 µg/mL of rifapentine as a stock-I solution. The calibration curve was prepared with a concentration ranging from 10 µg/mL to 50 µg/mL of rifapentine. For drug sample preparation, the tablet rifapentinefin (600 mg) was procured from Dr. Miltons Lab Pvt. Ltd., India, in tablet form. The power form (Eq.Wt for 10 mg = 18.16 mg) of the tablet was used for the analysis. The drug sample solution was prepared with methanol. 18.16 mg of rifapentinefin powder was dissolved in 10 mL of methanol to achieve a concentration of 1000 µg/mL of rifapentinefin (tablet solution-stock-II). 20 µg/mL of tablet solution-stock-II (0.2 mL) was used for the assay. The quality control samples were prepared with a 10µg/mL tablet solution in the mobile phase with concentrations of 8 mg/mL, 1 mg/mL, and 0.12 mg/mL, respectively. For accuracy testing, the percent recovery approach was established. %RSD was determined by the concentrations of 80, 100, and 120 percent of tablet and standard solution. For wavelength selection, 50µg/mL of rifapentine was prepared from the stock-I solution. Then the solution was scanned for 200–400 nm in a UV-visible spectrophotometer, and λmax was identified.

3. RP-HPLC method development and validation with QbD approach

Risk Assessment

A risk assessment was conducted to understand the target method's quality profile (TMQP) with the help of critical analytical attributes (CAAs). Risk assessment helps in understanding the individual risk factors. In the present study, three factors were considered for screening, namely, mobile phase, flow rate, and wavelength, with four responses such as RT (retention time), PA (peak area), TP (theoretical plate), and TF (tailing factor).

Optimization

The necessary trails were designed with a QbD approach. The BBD study employed the Design Expert version 13.00 tool to develop the response surface shape, which includes three independent components and four dependent elements. RT (retention time), PA (peak area), TP (theoretical plate), and TF (tailing factor) were examined as dependent variables after the optimisation of flow rate (mL/min), mobile phase MeOH (%) percentage, and wavelength (nm) as independent variables. A total of seventeen runs were done to achieve optimal chromatographic conditions.

Development of the method

After the customisation of chromatographic conditions, the RIF was identified and separated using a DAD

C-18 column at room temperature with a mobile phase flow rate of 1 mL/min and a run period of 5 minutes. The injection volume used was 20µL with mobile phase methanol and 1% OPA (81:19 v/v) under isocratic conditions. Elute was detected at 252 nm using the standard rifapentine. (Table 1)

Table 1: Coded values for independent variables

Name	Code	Low	High	Coded Values
MeOH comp (%)	A	80	82	-1, 1
Flow rate (mL/min)	B	1	1.2	-1, 1
Wavelength (nm)	C	251	253	-1, 1

Method validation

All validation measurements, including system suitability, linearity, LOD, LOQ, accuracy, precision, robustness, and stability, were assessed following ICH guidelines.

System suitability

To ensure repeatable findings, system suitability tests for equipment performance analysis were conducted in accordance with ICH guidelines by using API. Before assessing the sample batch, we evaluated the system's chromatographic reproducibility. Samples were tested for consistency, and %RSD was calculated based on three factors: retention time (RT), theoretical plate (TP), and tailing factor (TF).

Linearity

Rifapentine linearity was assessed in duplicates (n = 5) with different concentration levels ranging from 10 µg/mL to 50 µg/mL. The calibration curve was created by graphing the peak area against the concentration of a sample. The regression line equation and correlation coefficient (r2) were calculated.

Accuracy and precision

Three quality control tests were used to assess inter-day and intra-day precision: LQC (8µg/mL), MQC (10µg/mL), and HQC (12µg/mL) of API with less than 2% RSD acceptable limit at each concentration level. The accuracy test used the standard addition technique (percent recovery). The percent recovery and percent RSD were calculated for each concentration using the pre-estimated solution.

Robustness of the method

A robustness study was conducted to evaluate the effect of a small but purposeful change in chromatographic conditions. We measure retention time (RT), tailing factor (TF), and theoretical plate (TP) with methanol and water, flow rate, and wavelength.

RESULTS

The maximum absorbance of 50 µg/mL of rifapentine solution was observed at 252 nm. Therefore, 252 nm was the wavelength selected for further analysis. Primarily, the analysis with a mobile phase with MEOH to 1% OPA, 80:20 v/v, was done; however, no single peak was observed within the selected concentration. Then the mobile phase with MEOH to 1% OPA, 85:15 v/v, was tested, and improvements in peak shape and symmetry were observed. Further, the pH of the buffer was adjusted to 6 to achieve peak symmetry and shape. Then a mobile phase optimised to 89:19 v/v (MEOH to 1% OPA) with pH 6 achieved satisfactory optimisation of chromatographic conditions.

1. QbD approach for HPLC method optimization

The seventeen runs were used to analyse the response surface study type, central composition design, and quadratic design model. (Table 2)

Table 2: Optimization of parameters for analysis of Rifapentine

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A: MeOH comp (%)	B: Flow rate (mL/min)	C: Wavelength(nm)	RT	PA	TP	TF
1	80	1.1	253	4.892	1806.38	8164	1.25
2	81	1.2	251	4.195	1630.53	8028	1.12
3	81	1	251	4.331	1956.95	8693	1.06
4	80	1.2	252	3.821	1655.63	7675	1.17
5	81	1.1	252	4.084	1781.92	8121	1.36
6	81	1.1	252	4.067	1775.74	8259	1.16
7	81	1.1	252	4.065	1773.12	8260	1.16
8	81	1.1	252	4.066	1776.66	8262	1.26
9	82	1	252	4.342	1961.08	8526	1.06
10	80	1.1	251	4.516	1841.31	8023	1.02
11	80	1	252	4.86	1978.34	8695	1.14
12	81	1	253	4.781	2044.15	8417	1.11
13	81	1.2	253	3.889	1873.32	7834	1.25
14	82	1.2	252	4.86	1858.42	8421	1.16
15	82	1.1	253	4.785	1956.63	8249	1.13
16	82	1.1	251	4.975	1945.8	8546	1.16
1	81	1.1	252	4.28	1972	8193	1.27

The equation retention time (for actual values) RT1=4.08 + 0.1091 x A- 0.1936 x B + 0.0412 x C + 0.3893 x A x B - 0.1415 x A x C - 0.1890 B x C + 0.4221 x A^2 + 0.2504 x C^2 concluded that, as mobile phase MeOH increases, flow rate decreases, and the value of RT is increased. The p-values < 0.0001 indicate that the analytical model was significant. The signal-to-noise (S/N) ratio of 15.075, greater than 4, indicates a sufficient signal. Therefore, we interpret that this mode can help navigate the design space. (Figure no.1)

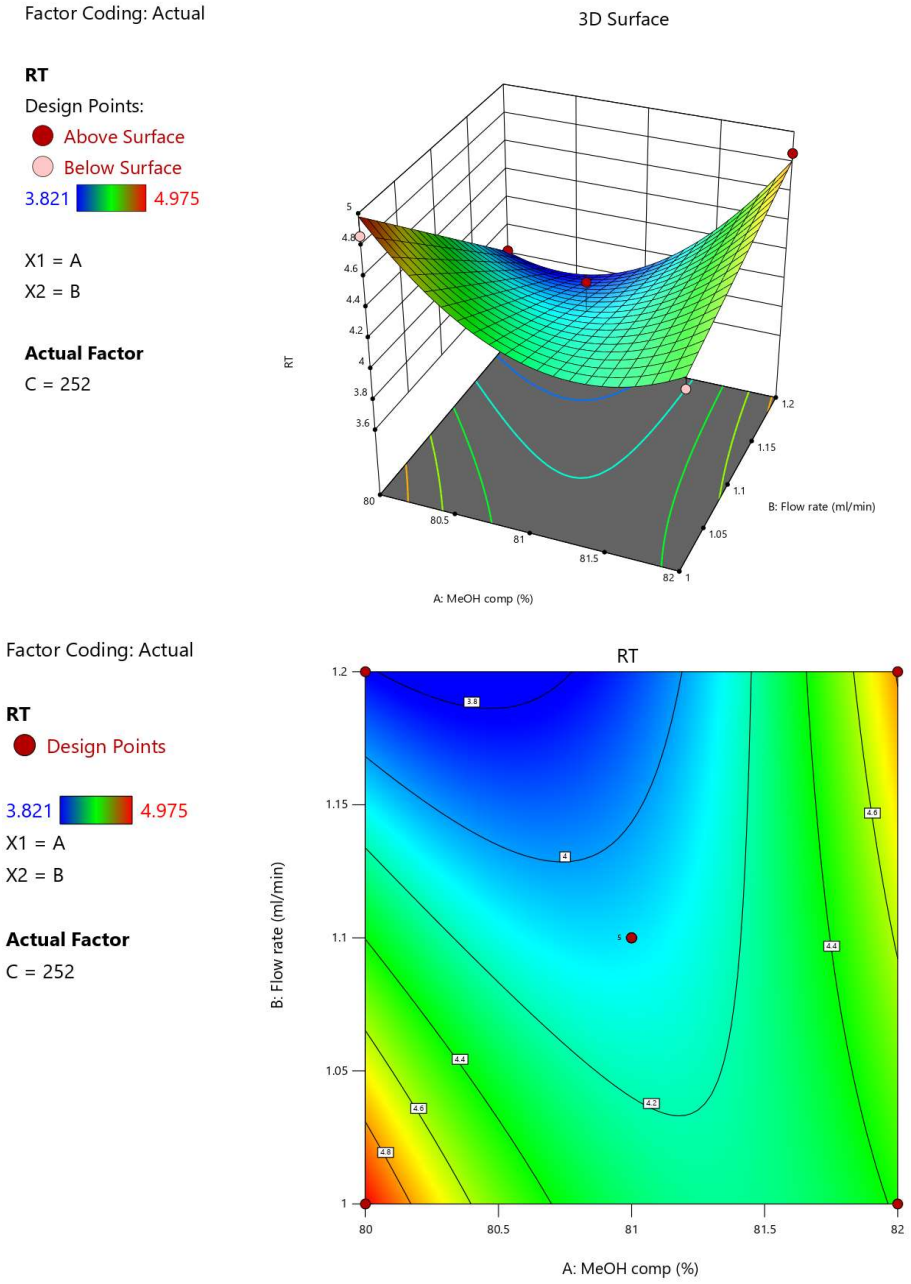


Figure no. 1: a) The effect of an independent factor on the retention time of a 3D sensitive surface. (b). Analysis of the independent factor's impact on retention time using a contour plot.

The equation peak asymmetry (for actual values) PA = 1858.12 + 55.03 x A - 115.33 x B + 38.24 x C interpreted that as mobile phase (A) increases (+55.03) with β2 (-115.33) flow rate decreases, the peak

asymmetry increases. The model F-value of 7.72 indicated that the model was significant; additionally, p-values < 0.0033 also indicated that the analytical model was significant. The signal-to-noise ratio of 8.958 indicates sufficient signal. Hence, the parameter was also optimised to direct the design space. (Figure no. 2)

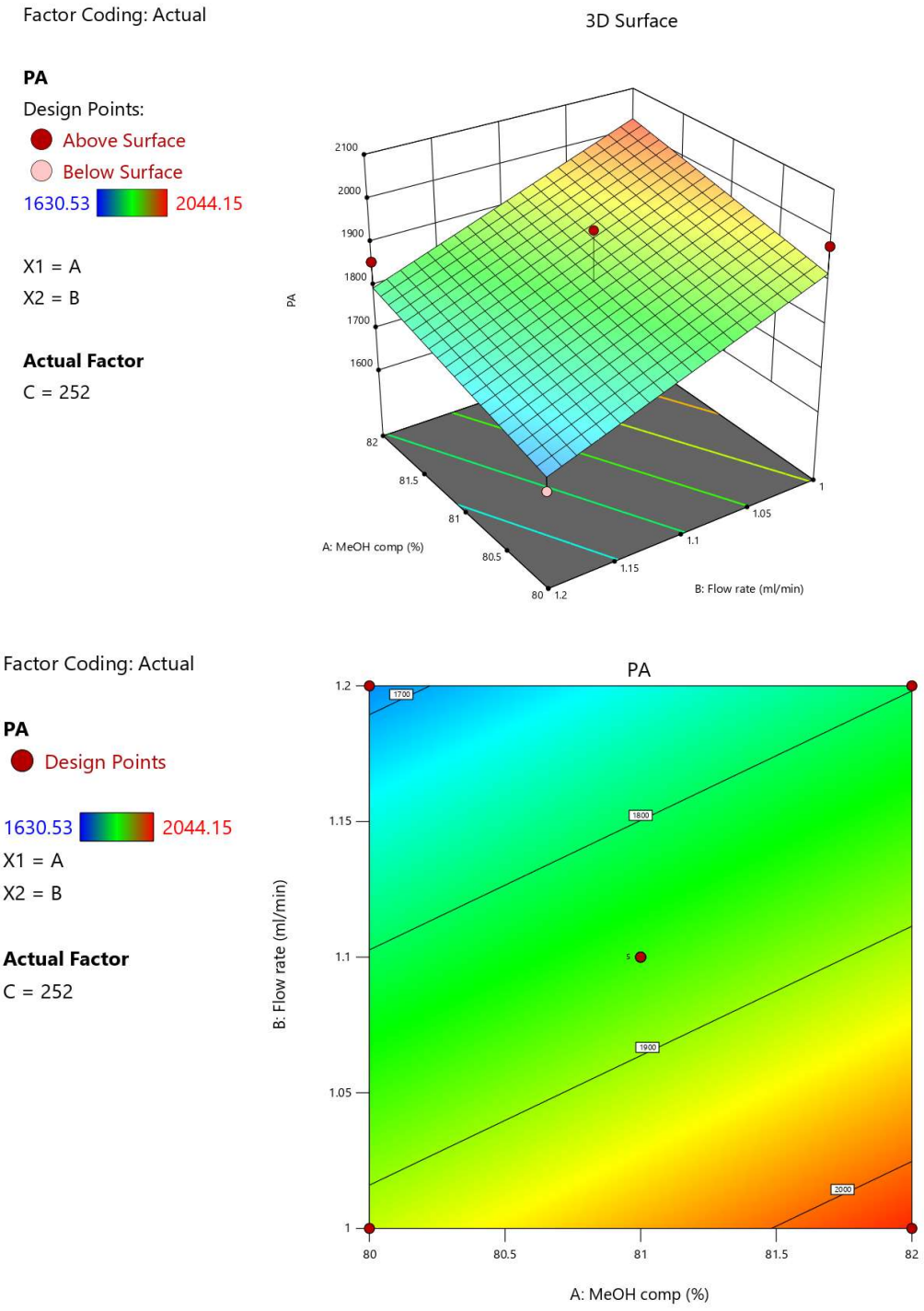


Figure no. 2: a) The effect of an independent factor on the peak area of a 3D sensitive surface. (b). Analysis of the independent factor's impact on peak area using a contour plot.

Moreover, the equation TP= 8256.82 + 148.13 x A -296.62 x B - 78.25 x C + 228.75 A x B - 109.50 x A

x C + 20.50 x B x C determined that mobile phase β1 positive coefficient (A, +148.13) increases, the β2 negative coefficient (B) decreased, and the value of TP is increased.

The model F-value of 33.45 indicated that the model was significant; moreover, p-values < 0.0001 also indicated that the analytical model terms were significant. The S/N ratio of 21.657 indicates a sufficient signal. Hence, this parameter was also optimised to navigate the design space. (Figure 3)

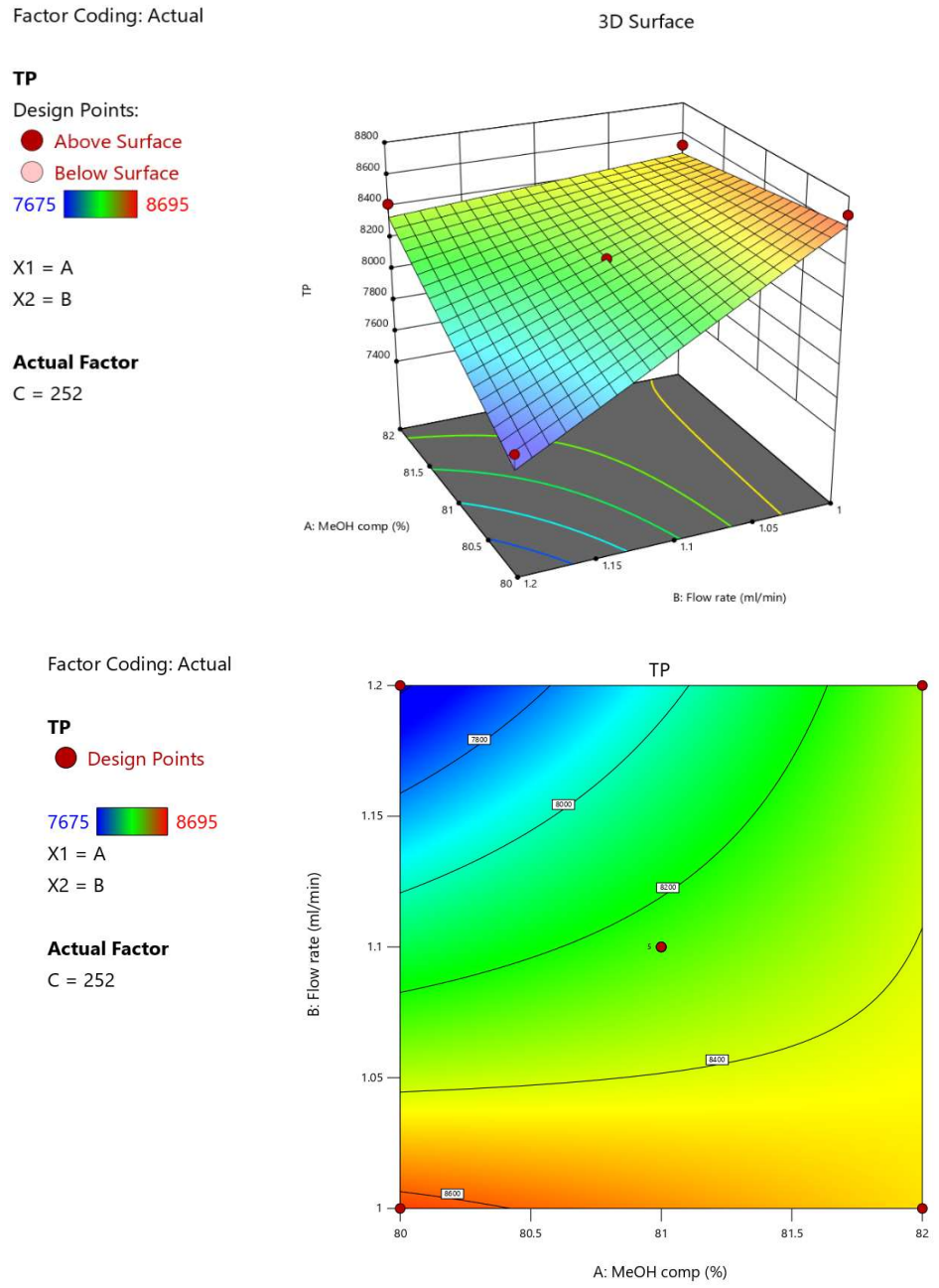
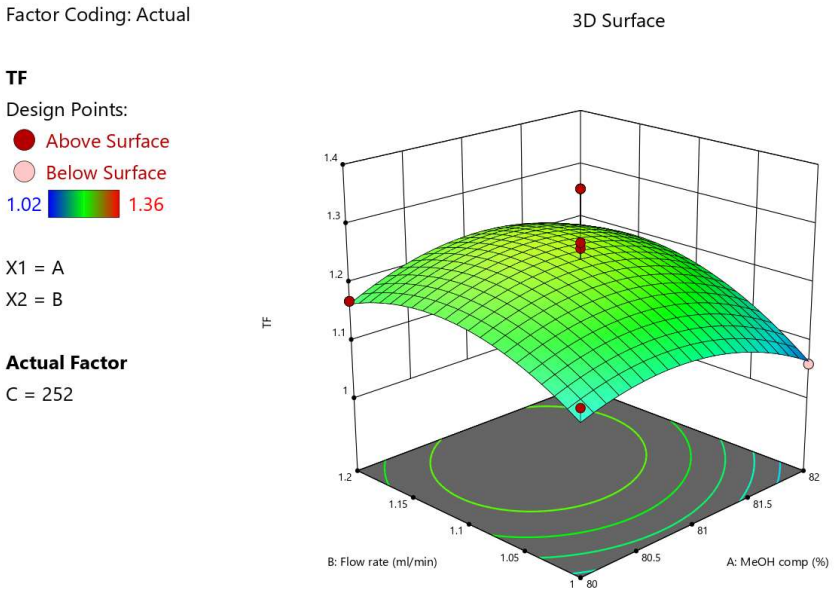


Figure no. 3: a) The effect of an independent factor on the theoretical plate of a 3D sensitive surface. (b). Analysis of the independent factor's impact on theoretical plate using a contour plot.
The equation for TF = + 1.24 - 0.0087 x A + 0.0413 x B + 0.0475 x C + 0.0175 x A x B - 0.0650 x A x C

+ 0.0200 x B x C - 0.0523 x A^2 - 0.0573 x B^2 - 0.0497 x C^2 resulted in the mobile phase coefficient decreased as flow rate and wavelength increased, and the TF increased. The model F-value of 2.33 implies the model was not significant relative to the noise. There is a 13.86% chance that an F-value this large could occur due to noise. (Figure 4); however, the S/N ratio of 4.433 indicates a sufficient signal. Hence, the parameter was also optimised to navigate the design space. (Figure 4).



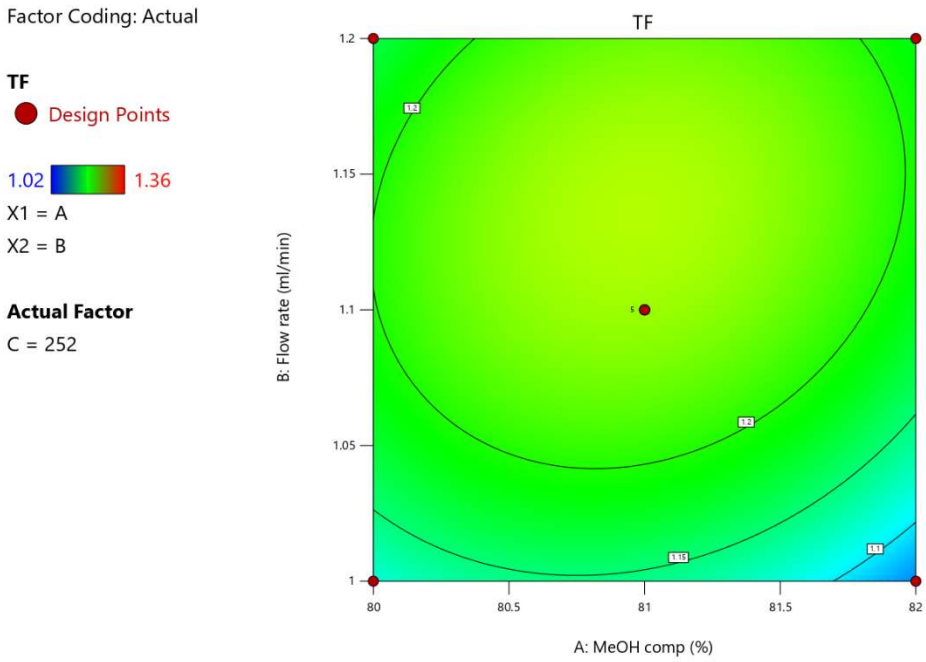


Figure no. 4: a) The effect of an independent factor on the tailing factor of a 3D sensitive surface. (b). Analysis of the independent factor's impact on tailing factor using a contour plot.

Thus, examining all the results, we have concluded that the surface sensitivity of BBD indicates that the experimental conditions were matched to linear and quadratic equations using multiple regression

2. Method validation

Linearity

The calibration curve for rifapentine showed linearity across the concentration range of 10 µg/mL to 50 µg/mL. (Figure 5, Table 3)

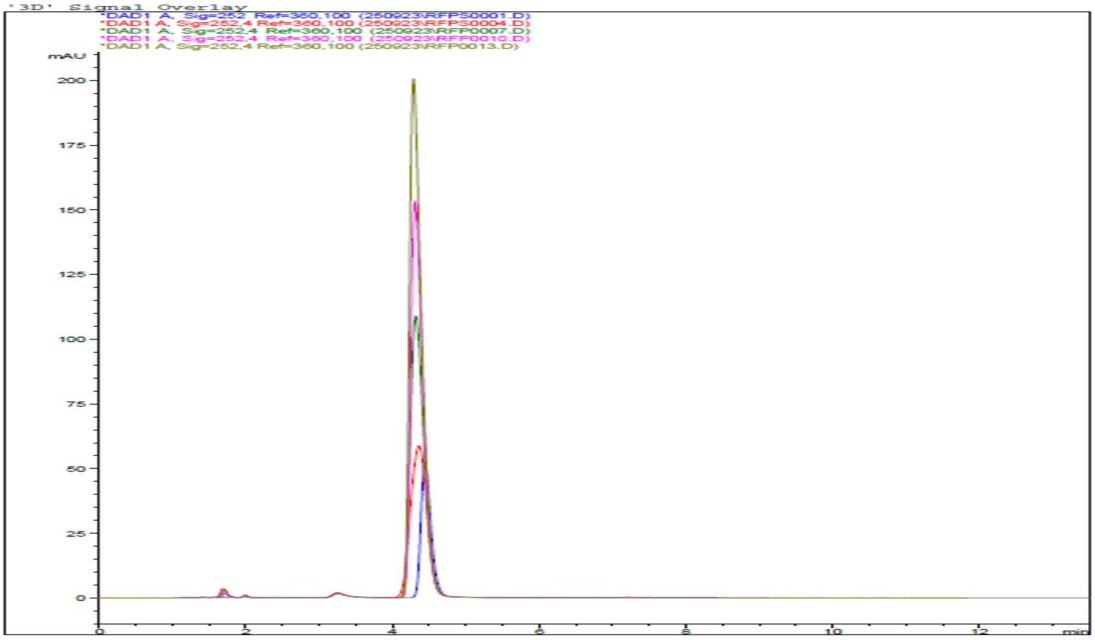


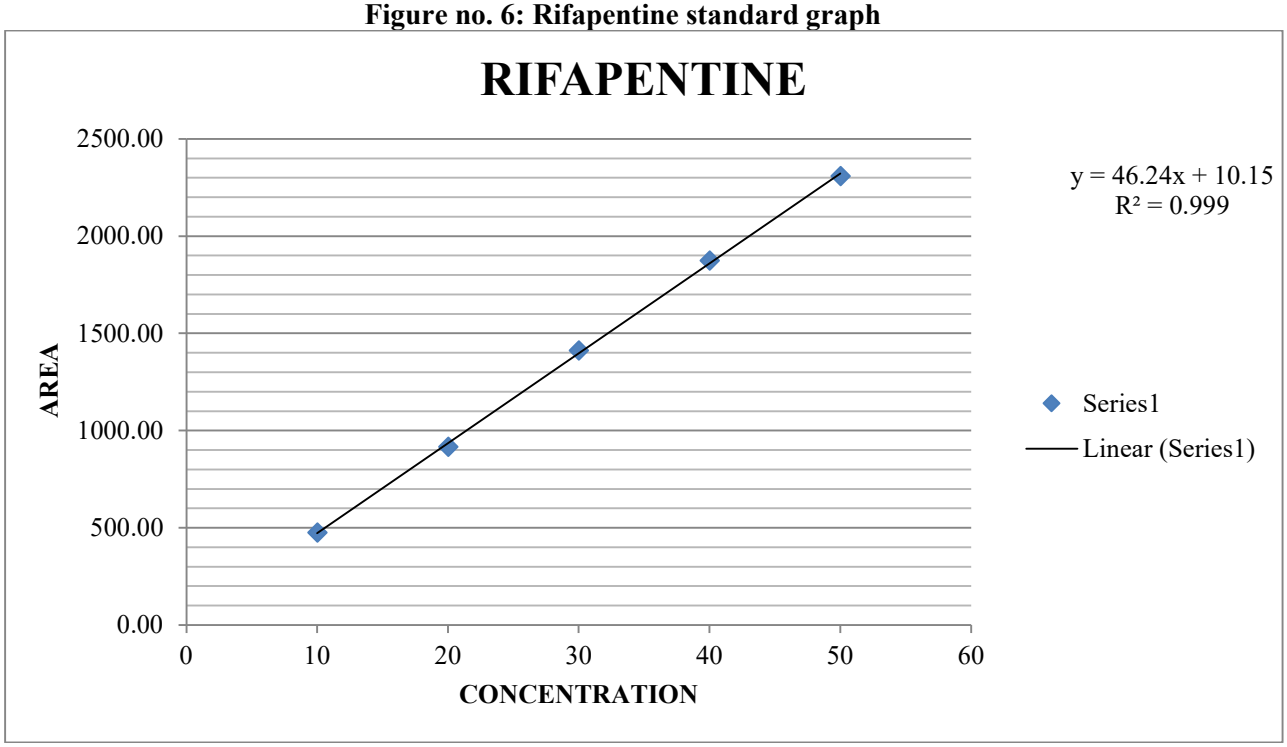
Figure no. 5: Linearity of 10 µg/ mL to 50 µg/mL of Rifapentine

Table 3: Linearity of Rifapentine

Concentration µg/mL (n=5)	Peak area (mean ± SD)
10	475.07±0.58
20	917.15±1.53
30	1412.19±2.18
40	1873.81±0.55
50	2308.83±1.51

The standard regression equation for the calibration curve was determined to be y = 46.24x + 10.15, with a correlation coefficient (R^2) of 0.999 when the peak area was plotted against concentration. (Figure 6)

Figure no. 6: Rifapentine standard graph



Precision

The %RSD for rifapentine repeatability was measured at a concentration of 50 µg/mL. The %RSD was found to be 0.0417, which was found to be under 2. Therefore, we concluded that the developed method demonstrated a high level of precision. (Table 4)

Table 4: Intraday and Inter-day of Rifapentine

	Concentration µg/mL (n=5)	(Mean ± SD)	%RSD
Intraday Precision	10	473.58±0.21	0.890
	30	1397.04±4.89	0.350
	50	2300.08±1.77	0.077
Intrer-day	10	477.04±1.59	0.890
	30	1405.29±1.58	0.113

Precision			
	50	2301.46±1.99	0.087

Accuracy

The recovery study was implemented to evaluate the method's accuracy. The sample solutions were examined (spiked) at 80%, 100%, and 120%. The recovery rates, ranging from 100% ±0.23 to 101% ± 0.39, confirm the high sample recovery according to the API. Therefore, we concluded that the developed method was accurate for rifapentine. (Table 5)

Table 5: The accuracy of the developed analytical method was evaluated with amount recovery study of the lead pharmaceutical ingredient

		Recovery rate for 80%			
Concentration µg/mL	**Amount added µg/mL**	**Area** mAU*s	**Amount found µg/mL**	**Amount recovered µg/mL**	**% Recovery**
10	8	844.84	18.0513	8.0512	100.6406
10	8	841.776	17.9849	7.9849	99.8123
10	8	842.7904	18.0069	8.0069	100.0866
		Mean	18.0144	20.5800	100.1798
		SD	0.0338	0.0338	0.4219
		%RSD	0.1876	0.1641	0.4212
		Recovery rate for 100%			
10	10	939.1972	20.0918	10.0918	100.9185
10	10	937.3679	20.0522	10.0522	100.5229
10	10	939.1821	20.09	10.0915	100.9152
		Mean	20.0785	20.5800	100.7855
		SD	0.0228	0.0228	0.2275
		%RSD	0.1134	0.1107	0.2257
		Recovery rate for 120 %			
10	12	1029.8220	22.0516	12.0516	100.4307
10	12	1034.1204	22.1446	12.1446	101.2057

10	12	1032.5926	22.1116	12.1116	100.9303
		Mean	22.1026	20.5800	100.8556
		SD	0.0471	0.0471	0.3929
		%RSD	0.2133	0.2291	0.3895

Robustness

The robustness of the method was evaluated using a 30 µg/mL rifapentine solution. The changes introduced changes in key parameters such as mobile phase, flow rate, and wavelength. The %RSD for the peak area remained below 2, despite variation. The %RSD was calculated as 0.13%, 0.18%, and 0.07% for the changes in mobile phase, flow rate, and wavelength, respectively, indicating the method's reliability.

The LOD and LOQ for rifapentine were calculated based on the SD of the slop and intercept and determined to be 0.091µg/mL and 0.27µg/mL, respectively.

Assay

The optimised chromatogram of rifapentine displayed a well-resolved peak with an RT of 4.399 minutes when the assay was conducted on the tablet. The % assay of the drug content was 99.23 ± 0.96 (n = 5) for the labelled amount of rifapentine. These results validate the method's accuracy and specificity, even in the presence of other pharmaceutical ingredients than the active compound found in the tablet powder.

DISCUSSION

TB is the leading cause of death globally, though the cure is available. However, the increased drug-resistant TB significantly challenges the treatment modalities. There are many TB medications available on the market, yet they are always questioned for their safety and efficacy. Hence, ensuring the safety and efficacy of the TB drugs produced is the top priority. QbD is a method validation approach now used in the modern pharma era for bulk pharma active ingredient production. In the current study, we optimised the RP-HPLC parameters such as mobile phase, flow rate, and wavelength, and with the QbD approach, method validation was achieved. The mobile phase combination, flow rate, and wavelength were recognised as the critical quality attributes influencing the analytical target product profile. Moreover, the CCD was utilised for three factors at four responses, such as retention time (RT), peak asymmetry (PA), theoretical plate (TP), and tailing factor (TF), by employing the Design Expert Software V.13.0.3.0. In the RP-HPLC method optimisation process, column selection, instrument configuration, and injection volume were kept constant, while factors such as mobile phase, flow rate, and wavelength were designated for the robustness study.

The present study successfully validated the RP-HPLC method for rifapentine, an anti-tuberculin drug, using a QbD approach. The optimised method determined for C18 (Fortis, 250 mm x 4.6 mm, 5.0 µm) and mobile phase combination of methanol to 1% OPA (82:19 v/v) with pH adjusted to 6. The current study was the first to use the different mobile phase combinations with methanol and 1% OPA. The RT for RPT was found to be 4.399 minutes at 252 nm. The method was found to be linear between concentrations of 10 µg/mL and 50 µg/mL, with a correlation coefficient of 0.999. The % RSD for repeatability, intra-day, and inter-day precision was found to be 0.0417, less than 2%, suggesting that the optimised method was precise. The LOD and LOQ were 0.091µg/mL and 0.27µg/mL, respectively, which determined the accuracy and robustness of the method. Moreover, the percentage recovery was found to be between 100% ±0.23 and 101% ± 0.39, as per the acceptance criteria of ICH guidelines. In 2014, Tahir et.al., used the mobile phase acetonitrile to 0.01M KH2PO4 buffer in ration 80:20 v/v to estimate RPT in bulk and pharma preparations. The author also concluded that the percent drug recovery was satisfactory, and the developed method was precise and accurate and could routinely be used.(17) Additionally, Chellini et.al., (2015) also developed and validated an HPLC method for RIF, INH, PZA, and EMB hydrochloride in pharmaceutical preparations. The author optimised the chromatographic condition with a mobile phase of 20 mM

monobasic sodium phosphate buffer with CAN. EMB detection was performed at 210 nm, and RIF, INH, and PYZ were detected at 238 nm using a DAD. Hence, the author proved that the method is specific, linear, precise, accurate, and robust and can be applicable to QC analysis in therapeutic formulations.(18) Moreover, Momin et al. (2017) have developed the HPLC method for the dry powder inhaler preparation of PA-824, moxifloxacin, and pyrazinamide for drug-resistance TB. The author used a Luna C18 column (150mm x 4.6mm, 100Å column) with a mobile phase combination of methanol and triethylamine phosphate buffer. The author interpreted that the assay was linear for concentrations ranging from 2.5 to 100 µg/mL, with LODs and LLOQs less than 2%. The recovery for the three anti-TB drugs was found to be between 99.6% and 106.8% with precision; therefore, the author concluded that the method was effective in evaluating the content of powder format even for inhalation use.(19) Plus, Bhattacharyya et.al., and Dhondage et.al., in 2022 has developed the RP-HPLC method for anti-tuberculin drugs in 2022. Bhattacharyya et.al., worked on the method of development of Bedaquiline, the new drug used in TB treatment. With acetonitrile and 0.1% trifluoroacetic acid as the mobile phase, the author proves the method's suitability, accuracy, and precision with deliberate changes in flow rate, wavelength, and injection volume.(16) Similarly, Dhondage et.al., have developed and validated the RP-HPLC method for RPT and Moxifloxacin hydrochloride in bulk ad tablet dosage form. With methanol to sodium phosphate dihydrate buffer in the in the mobile phase, the author presented the method validation. Linearity was studied in the concentration range of 4-24 µg/mL and 10–60 /per mL for RPT and Moxifloxacine hydrochloride, respectively, with a correlation coefficient of 0.999 and 0.998. Hence, the author interpreted that the method can routinely be used in QC in bulk and tablet dosage form.(19) Moreover, in 2023 and 2024, Staden et.al., and Fan et.al., presented the development and validation of HPLC methods for drug combination analysis. Staden et.al., worked on the first-line anti-tubercular drugs isoniazid, pyrazinamide, and rifampicin together with clofazimine. The author optimised the chromatographic conditions with 0.1% aqueous formic acid and ANC. The drug separation was achieved in 10 minutes, and method linearity, robustness, and precision were established, and hence the author proved the method validity and reliability.(20) Fan et.al., worked on the method development for a triple combination dry powder of pteromalid, moxifloxacin, and pyrazinamide. The author used the method demonstrated by Momin et.al., and proved its validity, accuracy, and precision for the separation of pteromalid, moxifloxacin, and pyrazinamide.(21) Above all, the literature evidence matches our outcome. The use of 1% OPA and methanol as the mobile phase was the first time achieved for the separation of rifapentine. Hence, the present method may be routinely used in bulk and pharmaceutical preparations.

CONCLUSION

The maximum absorbance for 50 µg/mL rifapentine was observed at 252 nm, which was selected for further analysis. Initial trials with a mobile phase of 80:20 (MEOH to 1% OPA) yielded no distinct peak. When adjusted to 85:15, improvements in peak symmetry were noted. Further optimisation was achieved with a mobile phase ratio of 81:19 and a pH of 6, producing satisfactory chromatographic conditions. The method validation demonstrated high precision, accuracy, and robustness, with linearity across 10–50 µg/mL (R2 = 0.999). The optimised assay for rifapentine in tablets showed a 99.23% drug content, confirming the method's reliability and specificity.

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