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STABILITY-INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF TRIMETHOPRIM: AN ANALYTICAL QBD APPROACH

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ABSTRACT

The purpose of this study is to present a novel approach for determining the concentration of trimethoprim (TMP) through the utilization of reverse-phase high-performance liquid chromatography (RP-HPLC) and a quality-by-design (QbD) method. TMP is commonly used to treat and prevent urinary tract infections (UTIs) such as cystitis. Previous research has shown a lack of a proven technique that accurately measures TMP over an extended period using RP-HPLC with analytical quality by design (AQbD) integration. To optimize the method, the analysis employed the Central Composite Design (CCD), allowing for the establishment of a relationship between various factors and responses to improve study accuracy. The separation of trimethoprim was achieved using a C18 column with dimensions of 250 mm in length, 4.6 mm in diameter, and 5 µm particle size. The column temperature was maintained at 35°C. An isocratic mobile phase of methanol with orthophosphoric acid buffer at pH 3.5 in a 60:40% volumetric ratio was used. The injection dose was 10 µL with a flow rate of 1.2 mL/min. Trimethoprim was identified by measuring its absorbance at 230 nm using a photodiode-array

detector. The strategy underwent validation following ICH Q14 Guidelines. The retention time of trimethoprim was 3.1 minutes. The linearity ranged from 3-10 µg/mL. The accuracy, reproducibility, and selectivity of the method were confirmed with an R^2 value of 0.9993. The approach also showed significant recovery under various conditions of forced degradation, demonstrating its ability to differentiate between trimethoprim and degradation chemicals. Overall, the method met all assessment criteria, confirming its credibility and reliability.

KEYWORDS: Trimethoprim; Analytical quality by design; HPLC; Method Development; Method Validation.

1. INTRODUCTION

Trimethoprim, an antibacterial medication, is scientifically known as 2,4-diamino-5-(3',4',5-trimethoxybenzyl) pyrimidine. It was originally synthesized by chemists Bushby and Hitchings.[1] This compound belongs to the class of diaminopyrimidines, which includes Trimethoprim (TMP). TMP acts as a dihydrofolate reductase inhibitor and is often used in combination with sulfonamide antibacterial drugs to enhance its effectiveness. The primary use of this medication is to prevent and treat urinary tract infections.[2] Commercially, trimethoprim is available in both single-dose and combined therapeutic compositions, specifically in the form of trimethoprim with sulfadiazine Tablets. The molar mass of this substance is 290.32 g/mol. It appears as a white or yellow-white powder and possesses moderate solubility in methanol, ethanol, and *n*-butanol, while being less soluble in water. Its mechanism of action involves inhibiting the activity of dihydrofolate reductase, an enzyme crucial for the production of folic acid, an essential component for constructing DNA.[3,4] Additionally, Trimethoprim displays efficacy against various harmful microorganisms. It is recommended to use Trimethoprim primarily for the treatment of simple, symptomatic urinary tract infections.[5] However, it can also be beneficial in treating infections caused by *Burkholderia pseudomallei*, *Aeromonas hydrophilla*, *Acinetobacter*, *Bartonella henselae*, *Moraxella catarrhalis*, *Brucella*, and *Mycobacterium TB*.

The combination of trimethoprim (TMP) and sulphonamides (SAs) is widely used in authorized premixes in the European Union.[6] This is mainly due to their strong antimicrobial properties, which make them effective in treating respiratory and gastrointestinal (GI) infections in livestock production such as cattle, sheep, and pigs. Various analytical techniques have been employed for the determination of trimethoprim, including square wave voltammetry.[7] Photo-Fenton Oxidation Technology.[8] extractive spectrophotometry.[9]

simultaneous spectrophotometry.[10] first-order derivative spectroscopy, UV-VIS spectrophotometry-colorimetry, spectrofluorometry, charge transfer complexes formation, and high-performance liquid chromatography [HPLC].[11,13]

Ensuring the safety, effectiveness, and quality of pharmaceutical products is crucial. Although there have been many literature reports on estimating TMP using liquid chromatographic methods, the effectiveness and reliability of these methods can be adversely affected by high variability and inconsistent performance.[14,15] To address these issues and ensure better method performance and reliability, Quality by Design (QbD) approaches have gained popularity in the development of analytical methods. Traditional optimization of HPLC (High Performance Liquid Chromatography) procedures typically involves analyzing one factor at a time while keeping other factors constant. This approach often results in a limited understanding of essential parameters. The QbD strategy overcomes the limitations of the traditional one-factor-at-a-time approach by allowing for a comprehensive study of multiple factors simultaneously along with the method responses. This approach greatly improves the ability to analyze and optimize the method, enhancing its overall effectiveness and ensuring the quality of pharmaceutical products.

According to the guidelines set forth by the International Council for Harmonization (ICH) in Q14, it is now mandatory to incorporate AQbD. Several researchers have examined the use of QbD principles in the development of analytical techniques. AQbD proves to be an effective strategy in creating and optimizing accurate and efficient analytical techniques while also minimizing costs. Implementing the QbD approach in developing analytical methods offers a more effective solution to Out-of-specification results and reduces the risk of method failure. In the field of pharmaceutical development, analytical methods hold significant importance, making the adoption of QbD for developing these techniques a logical and beneficial approach. This recommended strategy allows for regulatory flexibility, minimizes out-of-specification outcomes, ensures high reliability, and creates cost-effective analytical methods.

Factorial designs, like the BBD and CCD, are widely used in pharmaceutical analysis for conducting experiments. In this study, we employed the CCD method to optimize the HPLC method. The objective was to establish a relationship between independent variables, such as mobile phase flow rate and mobile phase ratio, and their corresponding responses, including retention time (RT), theoretical plate, and tailing factor. The developed method was then validated following the specifications outlined by the International Council for

Harmonization (ICH). Additionally, a degradation assessment was performed to evaluate the stability of the proposed HPLC method.

2. EXPERIMENTAL SECTION

2.1. Reagents and Chemicals

We obtained Trimethoprim from Azidus Laboratories Limited, situated in Chennai, India. Our procurement included 100 mg tablets manufactured by IPCA Laboratories Ltd. and sourced from the local market in Chennai. In order to guarantee superior quality, we acquired HPLC-grade methanol and AR-grade potassium dihydrogen phosphate from Sigma-Alrich.

2.2. Equipment's and Software

The study employed an HPLC system (Agilent) consisting of an 1100-class HPLC binary solvent delivery pump, a diode array detector, an autosampler equipped with a 20µL sample loop, and Empower software for monitoring and analyzing the resulting signals. To measure the pH of the solutions, an automated system called the electronic pH Meter 802 from Gujarat, India, was utilized. Additionally, an ultrasonicator from Maharashtra, India, was used to remove gases from the solvents. For data collection and analysis, the Design-Expert® version 12 software (Stat-Ease Inc., Minneapolis, USA) was employed. This software is commonly used in experimental design to optimize various chromatographic parameters and create a design space.

2.3. Chromatography Conditions

The HPLC technique was used to develop and analyze methods using isocratic separation. A C18 column measuring 250 mm x 4.6 mm x 5 µm was employed, and the column temperature was set to 350 °C. The mobile phase consisted of a mixture of methanol and dihydrogen orthophosphate buffer in a ratio of 60:40. The pH of the mobile phase was adjusted to 3.5 using a 1% OPA solution. The flow rate was set at 1.2 mL/min, and an injection volume of 10µL was used. Detection was done at a wavelength of 230 nm. The technique was validated following the guidelines set by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

2.4. Buffer and mobile phase preparation

3.5 grams of potassium dihydrogen orthophosphate (KH2PO4) were dissolved in 100 mL of HPLC-grade water. The solution was then diluted to a total volume of 1000 mL by adding additional water. The pH of the solution was adjusted to 3.5 using a 1% solution of ortho-

phosphoric acid. For the mobile phase, a mixture was prepared by combining 60 mL of HPLC- grade methanol with 40 mL of the KH2PO4 buffer in a 60:40 volume-to-volume ratio. To ensure the removal of any small particles, the buffer was filtered using 0.45 µ filters.

2.5. Preparation of trimethoprim standard stock solution

A concentrated solution of TMP was prepared by accurately weighing 10 mg of the drug. The weighed amount was immediately transferred to a standard flask with a capacity of 10 mL and dissolved using methanol. Subsequently, 0.1 mL of the solution was withdrawn and mixed with 10 mL of methanol, resulting in a solution with a concentration of 10 µg/mL.

2.6. Preparation of trimethoprim sample solution

After weighing twenty tablets, they were crushed into a powder, mixed, and dissolved in 10 mL of methanol with 10 mg of TMP. The solution was filtered through a 0.45-micron syringe filter, sonicated for five minutes, and finally diluted to a volume of 10 mL using a mobile phase solvent.

2.7. Solution stability

The trimethoprim solution was stored at room temperature and monitored for stability for a duration of 72 hours. We compared the percentage assay value of the reference sample with that of the freshly prepared samples.

2.8. Forced degradation study

Experiments were conducted to study the degradation of the trimethoprim stock solution. The samples were subjected to various stress conditions, including immersion in acid (1 M HCl) at room temperature for 1 hour, exposure to alkali (1 M NaOH) at room temperature for 12 hours, photolytic degradation in a UV chamber at 27 °C for 48 hours, and thermal degradation at 70 °C for 48 hours. Degradation was evaluated based on the appearance of additional peaks or a decrease in peak size. The extent of degradation was quantified by calculating the percentage of restoration. Acid Degradation: Prepare a 10 mL solution for acid studies by combining 1 mL of the stock solution with 1 mL of 1M hydrochloric acid (HCl). Store the mixture at room temperature for 1 hour. To initiate alkali degradation, mix 1 mL of the stock solution with 1 mL of 1 M NaOH in a standard flask. Allow the mixture to sit at room temperature for 1 hour before emulsifying it to obtain a final volume of 10 mL. Oxidative Degradation: In order to conduct oxidative experiments, create a 10% solution of H_2O_2 by mixing 10 mL of H_2O_2 with 100 mL of water in a standard flask. Take 1 mL of the

concentrated solution and mix it with 1 mL of the 10% H_2O_2 solution to achieve a final volume of 10 mL. Transfer the mixture to a standard flask and let it rest at room temperature for a period of 12 hours.

To study the solution's photolytic degradation, we transferred 1 mL of the original solution into a 10 mL standard flask and subjected it to UV radiation for 48 hours at a controlled temperature of 27 °C in a chamber. To analyze the thermal degradation, drug solutions of known quality were placed in an oven at 70 °C for 48 hours. Following this heat exposure, we injected the prepared solutions into the HPLC system using the most suitable chromatographic conditions.

2.9. Method Validation

The proposed method was verified in accordance with the guidelines outlined in ICH Q14.

2.10. Linearity, LOD, and LOQ

The stock solution of trimethoprim was divided into smaller portions, which were then transferred into 10 mL flasks. These aliquots were then diluted further using the diluent, resulting in concentrations ranging from 2 to 10 µg/mL. To analyze the linearity of the method, a calibration curve was plotted by correlating the concentration values with the corresponding peak areas. The linearity was evaluated by calculating the regression correlation coefficient and the intercept value. Additionally, the sensitivity of the method was determined by calculating the limit of detection (LOD) and the limit of quantification (LOQ).

3. RESULTS AND DISCUSSION

3.1. Method development assisted by AQbD

The AQbD approach was applied to develop a reliable and rapid RP-HPLC method for analyzing TMP in pharmaceutical formulations. **Figures 1 and 2 display the chromatograms of trimethoprim, illustrating both the standard and sample chromatograms**. The main objective was to determine the analytical target profile during method development, aiming for accuracy, precision, and efficiency. Through a series of preliminary tests, the essential variables and their impact on the corresponding outcomes were identified, leading to an improved method performance. When utilizing the AQbD approach for HPLC technique development, careful consideration of important variables and responses is crucial, requiring preliminary experiments and risk assessment**.**

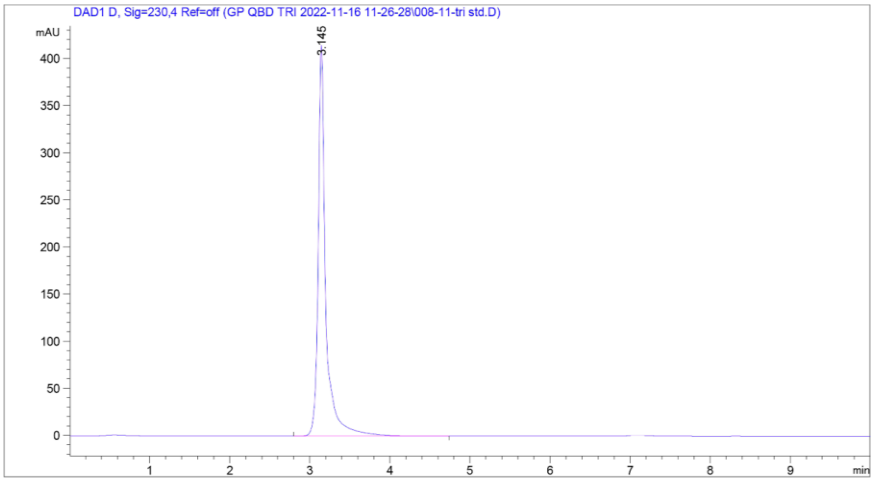

Figure 1: Chromatogram of standard trimethoprim.

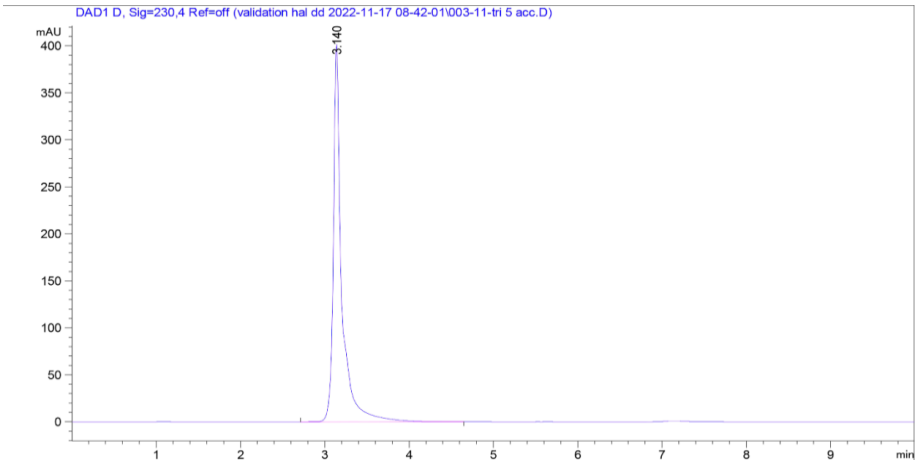

Figure 2: Chromatogram of sample trimethoprim.

3.2. AQbD aided method optimization

The Design of Experiments (DOE) methodology is used to determine the best composition parameters efficiently. This methodology helps identify significant impacts and their relationships. The Central Composite Design (CCD) is a crucial part of the Response Surface Methodology (RSM), which represents a quadratic response surface without a three-level factorial design. Our current research aims to optimize important parameters and empirical levels in the development of liquid chromatography technology, building on previous empirical investigations. In **(Table 1)**, we present the results of 13 experiments conducted on Trimethoprim. Each experiment had three central points and two parameters. The factor was concentrated in this range, with concentrations ranging from 50-60% v/v and flow rate variations of 0.8-1.2 mL/min. Through initial investigations, we found that optimizing the retention time of the peak concentration of trimethoprim, increasing the theoretical plate count, and achieving an ideal peak shape in response tests were effective strategies.

Table 1: CCD experimental design and measured responses.

		Factor 1	Factor 2	Response 1	Response 2	Response 3
Std	**Run**	**A:mobile phase**	**B:flow rate**	**R1**	**R2**	**R3**
				RT	Peak area	TP
12	1	55.0	1.00	2.6	2422	6876
3	2	51.5	1.14	2.2	1894	8865
4	3	58.5	1.14	2.1	1953	3266
1	4	51.5	0.86	2.9	2529	9876
10	5	55.0	1.00	2.7	2315	6756
11	6	55.0	1.00	2.8	2272	6999
6	7	60.0	1.00	2.4	2210	4011
2	8	58.5	0.86	2.8	2568	3789
5	9	50.0	1.00	2.5	1507	9987
9	10	55.0	1.00	2.9	2188	6889
7	11	55.0	0.80	3.1	2725	8810
13	12	55.0	1.00	2.5	2174	7856
8	13	55.0	1.20	2	1812	5890

3.3. Methods for optimizing chromatographic conditions utilizing CCD

Response surface methodology (RSM) was utilized to optimize a second-order model using a rotatable central composite design (CCD). CCD is a beneficial approach for sequencing research as it allows for determining axial positions and previous centers in factorial experiments on a regular basis. The chromatographic separation of trimethoprim involved parameter optimization, specifically the adjustment of variables such as the composition of the mobile phase (60–40% v/v methanol) and flow rate (0.8–1.2 mL/min) at five different levels. A total of thirteen experimental runs, including five center points, were conducted using CCD. The errors in each variable were measured five times at the zero level.

The responses were analyzed, and extraneous variables were eliminated from the model using a backward elimination technique to improve readability and relevance. The analysis of variance (ANOVA) results and descriptive statistics for the responses are provided in **Table 2**. Significant model terms were identified based on a *p* value threshold of 0.05. The polynomial terms showed *p* values less than 0.5, indicating their significant influence on the outcomes. The retention times for TMP were determined as 2.9 minutes.

Table 2: Summary of ANOVA and Regression Models.

Response	Model	F value	p- values	%CV	Precision (Adequate)	R^2 (Adjusted)	Std. Dev
Retention time	Quadratic	16.77	<0.0001	4.72	12.6524	0.8679	0.1261
Peak area	Quadratic	51.00	<0.0001	3.94	20.9179	0.8929	89.49
Theoretical plates	Quadratic	19.31	<0.0001	10.93	13.8733	0.8841	755.40

The R^2 values for three responses (A: 0.8679, B: 0.8929, and C: 0.8841) were greater than 0.8, ensuring that the selected quadratic model is suitable for data interpretation. Precision is crucial for advancing the model through the optimization process, and a value greater than 4 was attained for repeatability. At least four points in each response scored high for accuracy. The coefficient of variation (CV) was used to assess the need for model repetition, with a low CV indicating consistent results with minimal variation. The responses exhibited a lower coefficient of variation. Statistical methods, including analysis of variance, lack of fit (LOF), perturbation plots **Figure 3 (a-c)**, 2D contour **Figure 3 (D-F)**, 3D surface plots **Figure 3(G-I)**, and design space analysis were employed to analyze each response. The variance analysis indicated the significance of the experimental model.

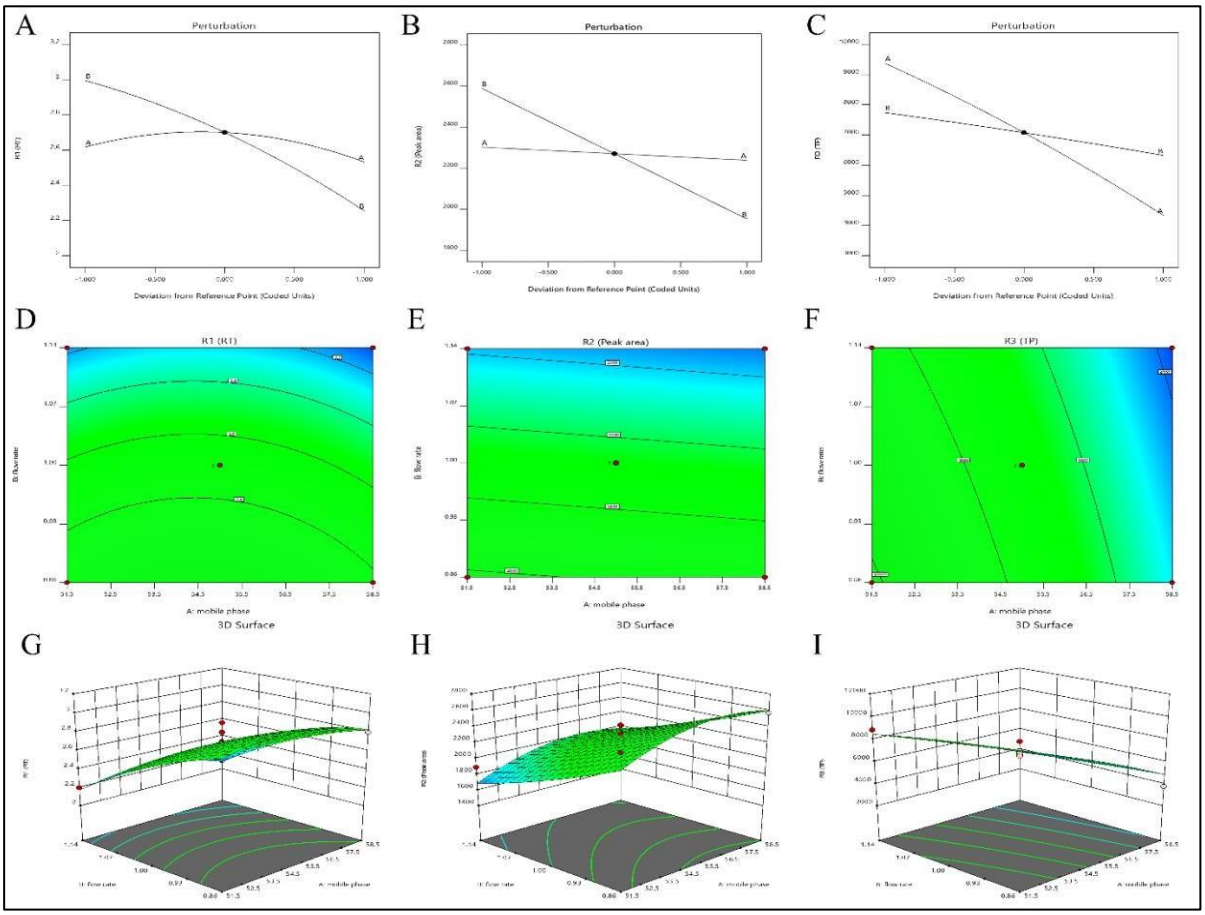

Figure 3: Perturbation plot (A-C), contour plot (D-F), and 3-D response plot (G-I) for retention time, Peak area, and theoretical plates of trimethoprim, respectively.

The model coefficients used in this analysis showed statistical significance, characterized by p- values below 0.0001. The Fischer-Ratios (F-value) quantified the importance of each coefficient in the model. A higher R2 value and lower lack-of-fit value confirmed good

model fit, while a high F ratio indicated the statistical importance of the analytical model equation. The impact of variables on each response was evaluated through the analysis of graphical data, including contour, perturbation, and 3D surface graphs. **Figure 4** depicts the overlay plot with optimum design space region. The statistical relationship between variables and responses is expressed by the polynomial equations.

R1 Retention time	=
-28.02365	
+1.08793	mobile phase
+4.88756	flow rate
-2.58487E-15	mobile phase * flow rate
-0.010000	mobile phase ^{2}
-3.75000	flow rate ^{2}
R2 peak area	=
+5013.96214	
-9.03518	mobile phase
-2246.10435	flow rate
R3 theoretical plates	=
+9347.31433	
+975.41751	mobile phase
-14028.37725	flow rate
+244.00000	mobile phase * flow rate
-17.55800	mobile phase ^{2}
-2198.75000	flow rate ^{2}

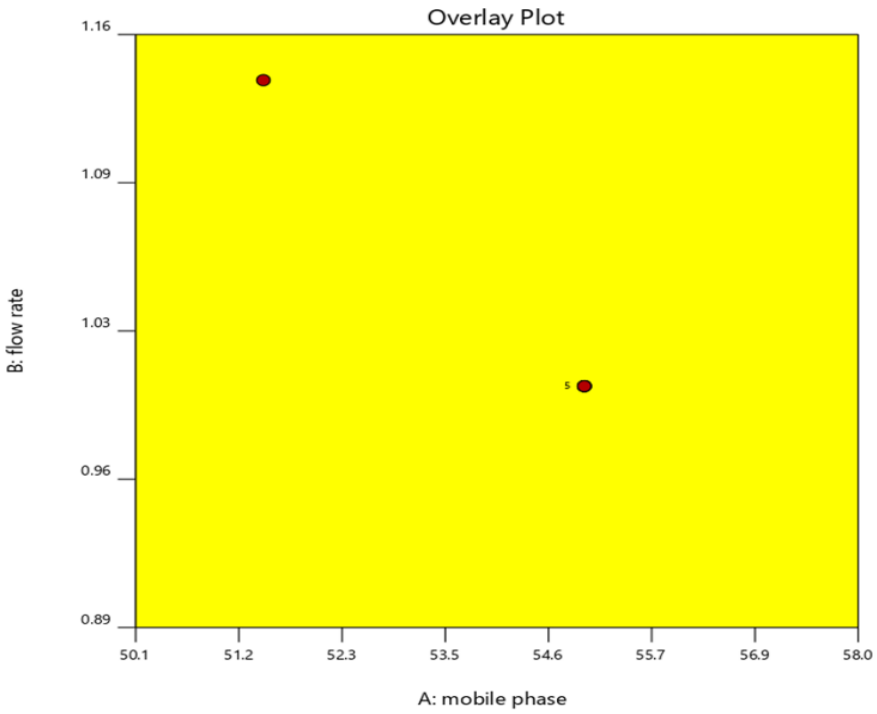

Figure 4: Overlay plot AQbD (method operable design region).

The Derringer's desirability function was used to optimize responses, considering different targets, with the aim of achieving suitable system suitability parameters for efficient peak separation. To enhance column efficiency, analysis time was reduced, and theoretical plates were maximized by minimizing peak retention time. Control methodology was established based on expected experimental conditions. System suitability parameters were calculated and achieved within acceptable limits. Anticipated errors were found to be negligible. This demonstrates that the model can be relied upon and replicated in a manufacturing environment to separate trimethoprim effectively.

3.4. METHOD VALIDATION

3.4.1. Linearity, LOD, and LOQ

There was a strong linear correlation between the concentration and peak areas of TMP within the range of 3-10 g/ml, as per the optimized chromatographic conditions. The correlation coefficient (R2) was found to be 0.9993, indicating a high degree of correlation. **Table 3** presents the linearity results, including the slope and intercept. By analyzing the standard deviation (SD) of the response and the slope of the regression line, the LOD and LOQ values were determined. The suggested method demonstrated high sensitivity, with an LOQ of 2.890697 µg/ml and an LOD of 0.95393. The LOQ and LOD determined by applying the following formulas complied with ICH regulations.

LOD = 3.3 × s/S LOQ = 10 × s/S

σ = the standard deviation of the response.

S = the slope of the calibration curve.

Table 3: Validation of analytical linearity parameters using HPLC.

Parameters	**Trimethoprim**
Concentration (µg/ml)	3-10
Regression equation	486.36x + 219.97
Correlation coefficient (R^2)	0.9993
Slope	486.36
Intercept	219.97
LOD (µg/ml)	0.95393
LOQ (µg/ml)	2.890697
Analysis of commercially accessible Trimethoprim	
Label claim	100 mg
Amount found	9.89 mg
% of Assay	99.48
% Recovery (w/w)	99.56

3.4.2. Precision and Accuracy

After conducting thorough investigations, the results of the precision tests are presented in **Table 4**. Since the relative standard deviation (RSD) did not exceed 2%, it can be concluded that the precision of the measurements was highly reliable.

The accuracy of the developed method was also evaluated, which revealed a wide range of percentage recoveries (% Recovery) in **Table 4**. These results indicate that the proposed technique exhibits exceptional precision in its measurements.

Table 4: Accuracy and Precision results for TMP.

Amount	Amount found	% Recovery	Mean	Repeatability % RSD
3	3.017	100.56		
3	3.011	100.36	100.41	0.125%
3	3.010	100.33		
4.2	4.096	97.52		
4.2	4.090	97.38	97.49206	0.101%
4.2	4.098	97.57		
6.5	6.326	97.32		
6.5	6.218	95.66	96.89	1.12%
6.5	6.350	97.69		

3.4.3. Solution stability

The analysis of the solution stability revealed no signs of degradation peak and no significant alteration in the peak area over a period of 72 hours, as observed from the obtained chromatograms. The test results showed a deviation of less than 2% compared to the initial fresh solution.

3.4.4. Forced degradation studies

Trimethoprim was subjected to forced degradation studies under different conditions, including 1 M HCl, 1 M NaOH, and thermal exposure at 70°C. These conditions were chosen to gain valuable insights into the stability of trimethoprim. The acid hydrolysis process revealed that approximately 21% of the trimethoprim samples underwent degradation, with peak area drastically reduced. No detectable degradation or peak was observed during the alkali hydrolysis process. No disintegration of trimethoprim was observed when exposed to light and heat in its solid state during the thermolytic and photolytic degradation process. The results of the degradation study, along with the corresponding chromatogram, are presented in **Table 5**.

Table 5. Forced Degradation Studies of Trimethoprim.

Description	% Recovery	Rt
STD	99.95	3.1
Acid (0.1 M HCl) – 24 Hrs	48.57	3.1
Alkali (0.1M NaOH) – 24 Hrs	98.05	3.1
Photo degradation – UV light	27.49	3.1
Thermal degradation	98.29	3.1

4. CONCLUSION

A highly efficient and optimized reversed-phase high-performance liquid chromatography method was successfully developed and validated for evaluating trimethoprim. This method adhered to the guidelines set by the International Conference on Harmonization. By employing the Analytical Quality by Design technique, specifically using the Central Composite Design method, the mobile phase percentage and flow rate were optimized. This AQbD approach not only allowed for the identification of method variables but also resulted in the development of stable and reliable techniques suitable for quality control laboratories without the need for additional revalidation. Interestingly, no additional peaks were detected during the degradation process, and even under suboptimal degradation conditions, there was no overlap of peaks. Overall, this method is highly advantageous, being both responsive and accurate, and can serve as a stability-indicating tool in various industries and regular quality control procedures.

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