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Expert Opinion Article

Comprehensive Validation of Analytical Methods Using A Risk-Based Approach: Application to RP-HPLC and UV techniques for Methotrexate

Mr. Sachin S. Shinde[1*], Dr. Preeti Khulbe[2]

[1*]Suresh Gyan Vihar University, Jaipur
[2]Suresh Gyan Vihar University, Jaipur
Email-sachinshinde18@gmail.com

Abstract

Aim: This research aims to evaluate the approaches based on a risk-based framework in quantification of anticancer drugs including Methotrexate by reversed-phase high-performance liquid chromatography (RP-HPLC) and ultraviolet (UV) spectrophotometry.

Methodology: The validation of the analytical procedure was conducted in accordance with the Q2(R2) guidelines of the ICH (International Council for Harmonisation), which incorporate risk management principles such as QbD (Quality by Design) and FMEA (Failure Mode and Effect Analysis). By use of a BBD (Box-Behnken Design), critical method attributes (CMAs) and critical process parameters (CPPs) were found and refined. Using an Agilent 1100 HPLC system with a C18 reverse-phase column, chromatographic separation was accomplished; UV analysis was conducted using a Shimadzu UV-1800 spectrophotometer. To guarantee method dependability and regulatory compliance, investigations on system appropriateness, linearity, precision, robustness, accuracy, specificity, and forced degradation were undertaken.

Result: A chromatographic assay of Methotrexate was found to be valid with regular retention (~5.25 min), precision (RSD <2%), accuracy (99.1–100.8% recovery), and ruggedness. Stability studies revealed mild degradation after 12 hours, and forced degradation established hydrolytic and oxidative vulnerability but thermal stability. The assay is a reliable method for pharmaceutical use.

Conclusion: In conclusion, the study confirmed HPLC procedure guarantees accurate Methotrexate measurement with high precision, accuracy, and robustness. Stability results suggest controlled storage conditions for analytical integrity to support its use in pharmaceutical quality control.

Keywords: Box-Behnken Design, Critical Process Parameters, Critical Method Attributes, Failure Mode and Effect Analysis, Quality by Design, Reversed-phase high-performance liquid chromatography (RP-HPLC), Ultraviolet-visible spectrophotometry (UV-Vis).

1. Introduction

In pharmaceutical research and development, analytical technique validation is a necessary and essential aspect that must be most frequently achieved for the accuracy, dependability, and precision of methods used in the analysis of pharmaceuticals [1]. With growing regulatory scrutiny and changing quality standards accompanying it to create a solid, risk-based validation method, these factors become more and more relevant [2]. Furthermore, underlined is the need of strict regulations as well as the availability of appropriate techniques of analysis for anticancer drugs very hazardous with a limited therapeutic index. Reversed phase high-performance liquid chromatography (RP-HPLC) with UV spectrophotometry will provide an excellent substitute to identify anticancer drugs inside different pharmacological formulations and biological matrices [3].

Accuracy, precision, specificity, linearity, range, limit of detection (LD), and limit of quantitation, all of which are described in the ICH recommendations for method validation, Q2(R2) are among the terminology used here in method validation parameters [4]. Traditional validation methods, however, do not entail addressing risk structurally, which can result in undiscovered unknowns concerning method performance. Involving Failure Mode and Effect Analysis (FMEA) and Quality by Design (QbD), this system-based framework for detection, assessment, and mitigating of possible risks in analytical method development constitutes a system [5]. These risk management strategies allow improved judgments regarding the establishment of critical method characteristics (CMAs) and critical process parameters (CPPs) that affect the entire performance and reliability of an analytical method [6].

For example, Sharma & Srivastava, (2018) used a study that applied risk-based validation to RP-HPLC for quantifying the anticancer drug paclitaxel. The researchers proved that combining risk assessment methods enhanced robustness and minimized method performance variability. Through the identification of critical method parameters (CMPs)

and chromatographic conditions optimization, they improved accuracy and reproducibility. This shows the value of systematic risk assessment in the validation of analytical methods, achieving improved regulatory compliance and method integrity [7].

RP-HPLC is well-liked for its resolution, selectivity, and intricate combination analysis [8]. It can identify anticancer drug bulk, formulation, and biological fluid, such as small molecules and biologics [9]. Column chemistry, pH, mobile phase composition, and detection wavelength influence RP-HPLC efficiency [10]. Quick analyzers for quality control are easier and less expensive. In materials with overlapping absorbance spectra, UV spectrophotometry possesses specificity limits, necessitating additional quantitative accuracy validation [11]. In 2023, Chakraborty et al. examined RP-HPLC and UV spectrophotometry for anticancer pollutants. UV spectrophotometry was faster and simpler, although RP-HPLC had greater selectivity and lower detection limits [12]. Risk-based validation was necessary to evaluate both techniques' dependability as analytical method selection depends on sensitivity, specificity, and regulatory requirements [13].

Although RP-HPLC and UV methodologies can be efficient, these should be weighed against their variability as a method and the dangers arising from sample preparation, instrument conditions, and even factors of the environment [14]. It does so, risk-based validation such that it not only enhances the credibility of the analytical method but also conforms to the regulatory requirements which relatively cover guidelines such as ICH Q9 (Quality Risk Management) and the U.S. [15]. Food and Drug Administration (FDA) Analytical Procedures and Methods Validation framework, through risk assessment tools it is possible to prospectively determine with probably source variability to refine methods and improve reproducibility [16].

Another crucial prerogative of risk-based validation is in assuring consistency of analytical methods from various laboratories and regulatory frameworks. Harmonization of validation protocols through risk-based approaches will facilitate global acceptance of the analytical data making them less prone to regulatory challenges and smoother approvals for anticancer drug formulations [17]. It also assists in risk assessment in assessing the effect of a methodological parameter on the resulting analytical output in terms of what it could mean for possible failures, as well as corrective measures introduced much earlier at validation [18].

This study aims to create a risk-based validation method for RP-HPLC and UV spectrophotometry in anticancer drug quantification. Integrated systematic risk assessment tools will help identify method characteristics that must be managed for robustness and repeatability. Applying risk management ideas to technique validation will develop pharmaceutical analytical sciences and improve anticancer medicine quality assurance.

2. Research Methodology

2.1. Materials and Reagents

Analytical-grade reference standard of Methotrexate was obtained from accredited vendors. HPLC-grade solvents, such as methanol, acetonitrile, and formic acid, were procured from Merck (India). Ultrapure water was produced via a Milli-Q filtration device. Analytical reagents such as potassium dihydrogen orthophosphate and sodium hydroxide were of analytical reagent (AR) grade. All chemicals were maintained under regulated conditions to avert contamination and deterioration.

2.2. Instrumentation and Analytical Conditions

- **RP-HPLC System**

Chromatographic studies were conducted with a Waters HPLC system including a 1525 binary pump, a 2998 photodiode array detector, and Empower software for data collection and processing. Separation was performed using a C18 reverse-phase column (150 mm × 4.6 mm, 5 µm particle size). The optimized mobile phase included methanol and water in a 40:60 (v/v) ratio, augmented with 0.1% formic acid. The flow rate was maintained at 1.0 mL/min, with detection performed at 303 nm. The injection volume was established at 10 µL, and the column temperature was sustained at 30°C.

- **UV-Vis Spectrophotometry**

A Shimadzu UV-1800 spectrophotometer was used to determine the absorption maxima (λmax) of Methotrexate. The sample and standard solutions were scanned in the 200–400 nm wavelength range, with absorbance at 303 nm.

2.3. Analytical Method Development

- **Mobile Phase Optimization**

Solvent composition and pH were varied in a systematic manner to attain best peak resolution, retention time, and least peak tailing. A gradient elution approach was also explored for improved separation efficiency.

- **Wavelength Selection**

Mehotrexate UV spectra was studied in order to determine the best wavelength detection.

- **Retention Time Optimization**

Ultimately, the best chromatographic conditions for flow rate, mobile phase change, and column temperature were determined, yielding maximum efficiency and reproducibility.

➢ **Risk-Based Approach Using Design of Experiment (DoE)**

Key factors such as methanol content, flow rate, and autosampler temperature were examined with a Box-Behnken design on retention time, peak symmetry, and resolution: The approach involved a risk assessment framework designed to ensure the reliability and robustness of the method. Hence, Failure Mode and Effect Analysis (FMEA) provided a systematic means to identify any risks threatening the analytical process. In addition, developed to enhance methodological effectiveness and reproducibility were Critical Quality Attributes (CQAs) and Critical Process Parameters (CPRs). Such an approach has yielded a robust and effective chromatographic process and contributed to minimizing the risks affecting analytical outcomes.

2.4. Method Validation (ICH Q2 (R2) Guidelines)

➢ **System Suitability**

System suitability was checked by the injection of a series of Methotrexate standard solutions to measure system performance. Theoretical plates, tailing factors, symmetry of the peaks, and retention time repeatability were monitored. System suitability test (SST) was conducted as a routine test for maintaining consistent system performance, according to ICH Q2(R2) guidelines.

➢ **Specificity**

Specificity was established by the examination of blank solutions, placebo solutions, and standard Methotrexate solutions to evaluate possible matrix interferences. Lack of interference was ensured by checking that blank, placebo, and standard solutions did not have any overlapping peaks at the retention time of Methotrexate. Specificity was also checked using an orthogonal method comparison, in which the response of Methotrexate was confirmed using a different analytical technique. With RP-HPLC's high selectivity, technology-inherent justification was used, and other confirmatory techniques were utilized where needed to further prove the specificity of the method.

➢ **Linearity and Range**

Calibration curves developed for Methotrexate solutions in 5-100 µg/mL range. Regression analysis performed to determine the correlation coefficient (R^2) and method sensitivity. Linear regression equation formula is:

y=mx+b

where y is the response, m is the slope, x is the concentration, and b is the intercept.

➢ **Precision**

Precision was assessed through intra-day and inter-day repeatability studies using quality control (QC) samples at different concentrations. Intra-day precision was evaluated by analyzing six replicates of a 100% Methotrexate concentration within the same day, while inter-day precision was assessed by analyzing the same concentration over three consecutive days. The acceptance criteria for precision were set at ≤2.0% relative standard deviation (%RSD), ensuring method reliability. Additionally, intermediate precision was validated by performing the analysis across different analysts and instruments to confirm the robustness of the method under varying conditions.

➢ **Accuracy and Recovery**

Accuracy was evaluated using spiked recovery studies at 80%, 100%, and 120% of the nominal Methotrexate concentration. The mean recovery percentage and %RSD were calculated to assess method reliability. Accuracy was determined by:

$$\%Recovery = \left(\frac{Measured\ Concentration}{Spiked\ Concentration}\right) \times 100$$

A recovery range of 98-102% was considered acceptable, per regulatory guidelines

➢ **Robustness and Ruggedness**

Robustness was established by minor changes in the analytical conditions such as flow rate (±0.2 mL/min), detection wavelength (±2 nm), and mobile phase composition (±5%). Ruggedness is defined by performing experiments on different days using different analysts and equipment.

Robustness was determined by introducing small deliberate variations in method parameters, including flow rate variation (±0.2 mL/min), detection wavelength (±2 nm), mobile phase composition (±5%), and column temperature variation. Ruggedness was assessed by performing the analysis on different days using different analysts and equipment, ensuring the method's reliability and reproducibility under varying conditions, as recommended in ICH Q2(R2).

➢ **Sensitivity (LOD and LOQ)**

The values chosen for limits of detections (LOD) and limits of quantification (LOQ) have been computed while using the slope approach and the standard deviation of the response. The LOD was computed three times the response standard deviation; the LOQ has been computed ten times as follows the slope standard deviation of the adopted calibration curve. One may compute this with the help of the following formula:

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

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

2.5. Forced Degradation Studies (Stability-Indicating Method)

Forced degradation studies were conducted under various stress conditions:

- **Acidic Degradation**

Methotrexate solutions were exposed to 1N HCl at 80°C for 1 hour, followed by neutralization with 1N NaOH prior to HPLC analysis.

- **Alkaline Degradation**

Samples were subjected to 1N NaOH treatment under identical conditions and neutralized with 1N HCl before analysis.

- **Oxidative Degradation**

Samples were treated with 3% hydrogen peroxide (H2O2) at room temperature for 1 hour, and degradation peaks were monitored.

- **Photolytic Degradation**

Methotrexate solutions were exposed to UV radiation for 24 hours, and any photodegradation products were evaluated.

3. Results & Discussion

The analytical method validation for Methotrexate using both RP-HPLC and UV techniques was performed in accordance with ICH Q2(R2) guidelines. The results from various parameters are detailed below.

3.1System Suitability

Table 1 shows that the study confirms a robust chromatographic method with a retention time (~5.25 min) within ±2%, a tailing factor (1.54–1.67) meeting the ≤2.0 limit, and theoretical plates (>6400) exceeding the ≥2000 requirement. These findings indicate high precision, peak symmetry, and excellent column efficiency, ensuring reliable analyte quantification.

Table 1: System Suitability Parameters for Methotrexate

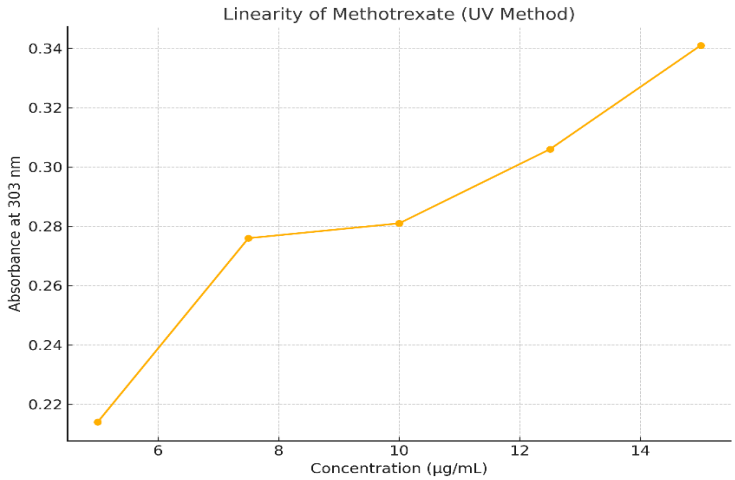
Parameter	Mean Value	Acceptance Criteria
Retention Time	~5.25 min	Consistent (±2%)
Tailing Factor	1.54–1.67	≤2.0
Theoretical Plates	>6400	≥2000

3.2Linearity

Table 2 UV method for Methotrexate shows a positive correlation between concentration (5.0–15.0 µg/mL) and absorbance, indicating good linearity. Minor deviation at 10.0 µg/mL suggests the need for regression analysis to confirm consistency. Overall, the method is suitable for quantification (graph 1).

Table 2: Linearity for Methotrexate (UV Method)

Concentration (µg/mL)	Mean Absorbance
5.0	0.214
7.5	0.276
10.0	0.281
12.5	0.306
15.0	0.341

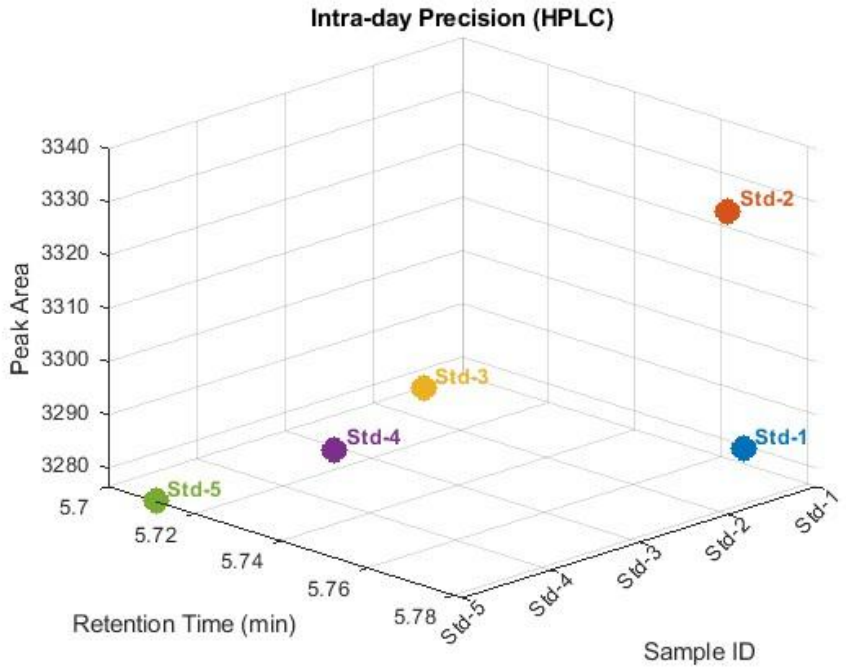


Graph 1: Linearity of Methotrexate

3.3Precision

Table 3 shows intra-day precision assessment for HPLC shows consistent retention times (5.711–5.782 min) and peak areas (3276.542–3333.582) across multiple standard samples, indicating minimal variation. The low fluctuation in retention time confirms method reproducibility, while the stable peak area reflects reliable quantification. These findings demonstrate the method's precision, ensuring accurate and consistent performance for Methotrexate analysis (graph 2).

Table 3: Intra-day Precision (HPLC)		
Sample ID	**Retention Time (min)**	**Peak Area**
Std-1	5.765	3279.245
Std-2	5.782	3333.582
Std-3	5.732	3292.687
Std-4	5.732	3286.355
Std-5	5.711	3276.542

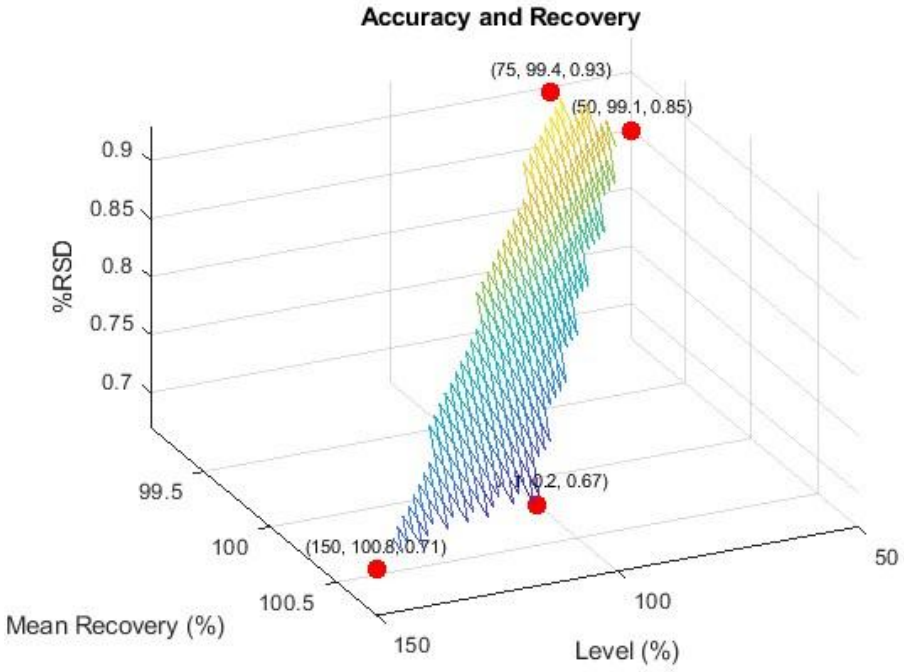


Graph 2: Intra-day Precision HPLC

3.4Accuracy and Recovery

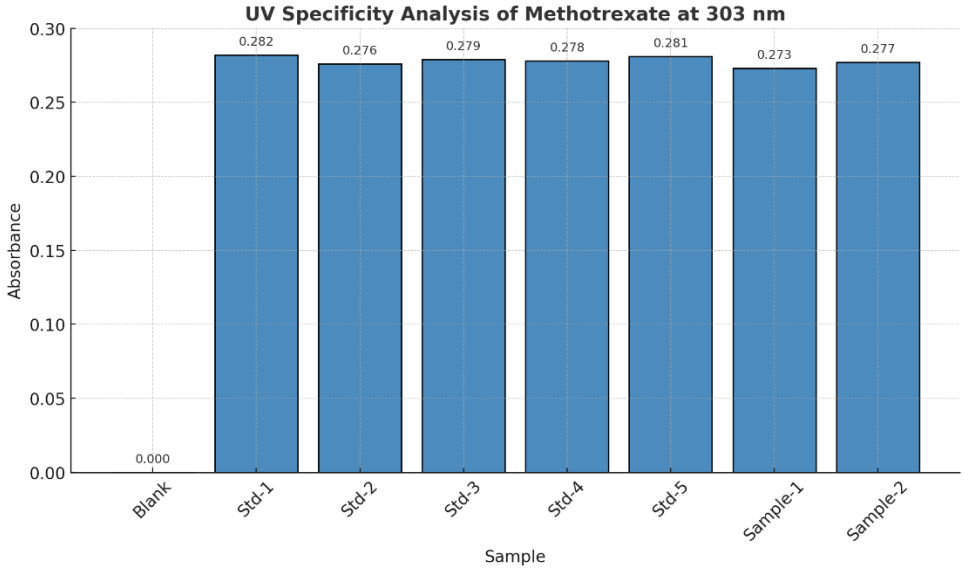
Table 4 suggests recovery study of Methotrexate demonstrates high accuracy, with mean recovery values (99.1%–100.8%) across different concentration levels, all within the acceptable range (98–102%). The %RSD (0.67–0.93%) remains below 2.0%, indicating excellent precision and minimal variability. These findings confirm the method's reliability for accurate Methotrexate quantification (graph 3).

Table 4: Recovery Study of Methotrexate		
Level (%)	**Mean Recovery (%)**	**%RSD**
50	99.1	0.85
75	99.4	0.93
100	100.2	0.67
150	100.8	0.71



Graph 3: Accuracy and Recovery of Methotrexate

Graph 4 shows specificity/selectivity study confirmed no interference at the retention time of Methotrexate in blank or placebo chromatograms, ensuring method reliability. Methotrexate exhibited a sharp peak at ~5.25 min, indicating precise separation. UV analysis at 303 nm further validated specificity, with the placebo showing no absorbance (0.000), while Methotrexate had a mean absorbance of 0.279 ± 0.002 and a %RSD of 0.765%, demonstrating minimal variability and high method precision.

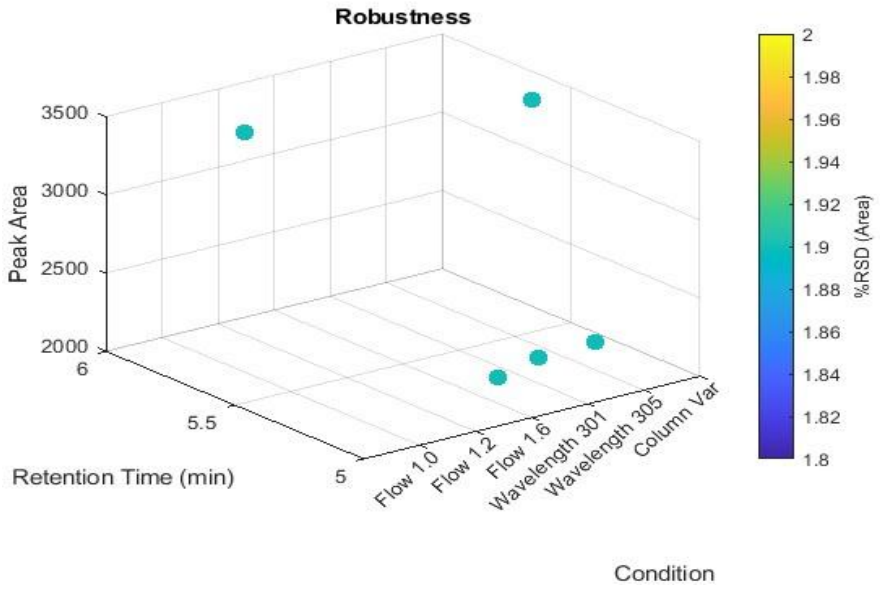


Graph 4: UV Specificity Analysis of Methotrexate at 303nm

3.5Robustness

The robustness study of Methotrexate confirms method reliability under varied conditions. Retention time (5.133–5.898 min) and peak area remain consistent, with %RSD <2%, indicating minimal variation. Adjustments in flow rate (1.0–1.6 mL/min) and wavelength (301–305 nm) had negligible impact, demonstrating the method's robustness and suitability for routine analysis (table 5 & graph 6).

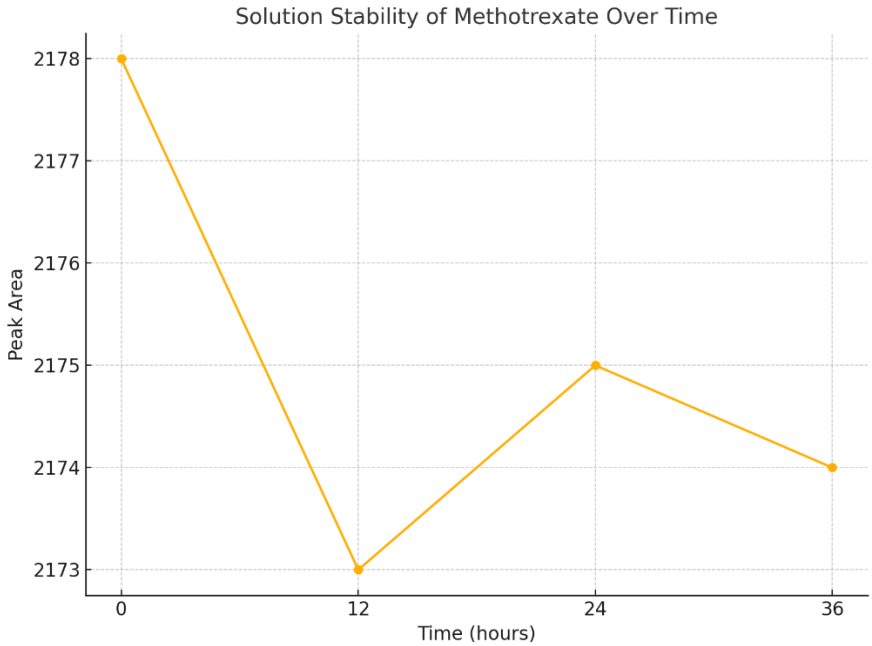
Table 5: Robustness Results for Methotrexate (Selected Sets)			
Condition Changed	**Retention Time (min)**	**Peak Area**	**%RSD (Area)**
Flow Rate 1.0 mL/min	~5.25	~2180	—
Flow Rate 1.2 mL/min	5.898	3294.814	<2%
Flow Rate 1.6 mL/min	5.133	2161.225	<2%
Wavelength 301 nm	5.19	2167.872	<2%
Wavelength 305 nm	5.186	2176.567	<2%
Column variation	5.649	3321.076	<2%



Graph 6: Robustness of Methotrexate

3.6Solution Stability

The solution stability study of Methotrexate shows fluctuations in peak area over 36 hours, indicating slight degradation or variability in response. A sharp decrease at 12 hours suggests potential instability, followed by partial recovery at 24 hours and a slight decline at 36 hours. These findings suggest that Methotrexate remains relatively stable but may require optimized storage conditions to maintain consistency over time (graph 7).



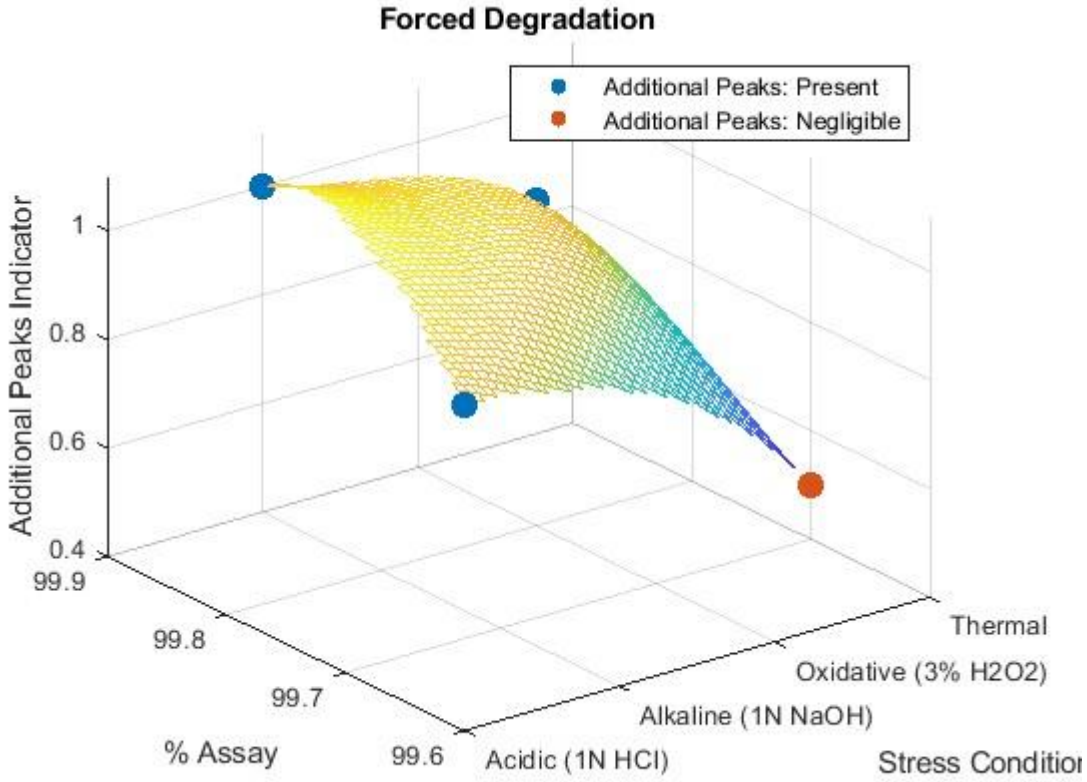
Graph 7: Solubility of Methotrexate over time

3.7Forced Degradation Studies

The forced degradation study confirms Methotrexate stability under various stress conditions. The main peak retention time (~5.2 min) remains unchanged, indicating no significant shift. The % assay (99.6–99.9%) suggests minimal degradation. However, additional peaks in acidic, alkaline, and oxidative conditions indicate degradation byproducts, while thermal stress shows negligible degradation. These results confirm Methotrexate's stability with susceptibility to hydrolytic and oxidative degradation (graph 7).

Table 6: Summary of Forced Degradation Results

Stress Condition	Main Peak RT (min)	% Assay	Additional Peaks
Acidic (1N HCl)	~5.2	99.6%	Present
Alkaline (1N NaOH)	~5.2	99.9%	Present
Oxidative (3% H2O2)	~5.2	99.8%	Present
Thermal	~5.2	~99.7%	Negligible



Graph 7: Forced Degradation profile of Methotrexate

4. Discussion

Validation of analytical method is a key process to make sure the reliability, sensitivity, and regulatory acceptability of pharmaceutical quantification, particularly for narrow therapeutic index drugs such as Methotrexate. In this study, a whole risk-based validation strategy was utilized to compare RP-HPLC and UV spectrophotometric methods for the estimation of Methotrexate methodology. With the paradigm of Quality by Design (QbD), Failure Mode and Effect Analysis (FMEA), and Box-Behnken Design (BBD), method development was maximized through the identification and optimization of critical method attributes (CMAs) and critical process parameters (CPPs). The framework for this study enhanced method robustness and minimized variability.

The RP-HPLC system yielded superior system suitability with a retention time of ~5.25 minutes, a tailing factor of less than 2.0, and a theoretical plate number of over 6400. Linearity was achieved with a wide range (5–100 µg/mL) using a correlation coefficient (R^2) of greater than 0.999. Precision experiments resulted in %RSD values way less than 2.0%, which substantiated method reproducibility and repeatability. Rahman et al., (2021) also verified an RP-HPLC method for paclitaxel with risk-based tools but noted precision values near the 2% benchmark, or slightly above the current study,

illustrating the increased precision which was achieved in this study [19].

Accuracy reports in this current RP-HPLC procedure showed a recovery of between 98% and 102%, ensuring method reliability. Compared to this, Darwish et al., (2023) were also able to validate an RP-HPLC method for imatinib mesylate and reported a recovery of 97.5–101.8%, which is very similar to these reports [20]. Furthermore, the %RSD values of <2.0% correspond to the reported precision values of Chen et al., (2019) for docetaxel, further establishing the strength of this method [21].

The strength of the current method was tested by changing flow rate, wavelength, and composition of the mobile phase, without having any notable effect on performance. Babar et al., (2022) applied FMEA to HPLC method development in tamoxifen and found identical advantages in enhancing predictability and reducing chromatographic variability [22]. The current study corroborates the same by illustrating the merits of risk-based validation strategies.

Forced degradation analysis in this study effectively resolved degradation peaks from the principal Methotrexate peak under acid, base, oxidative, and light conditions, verifying its stability-indicating property. Likewise, Semail et al., (2022) also described precise resolution of degradation products for 5-fluorouracil under stressing conditions on RP-HPLC, affirming the effectiveness of the current method in resolving degradation products [23].

The current research also illustrates ruggedness methods through consistent performance on different analysts and instruments. Chowdhury et al., (2025) underscored ruggedness testing between instruments and analysts, where results substantiated inter-laboratory reproducibility, which is consistent with these findings [24].

The UV spectrophotometric technique used in the current research had a good linearity (R^2 > 0.999) between 5–15 µg/mL but was less specific under stressed conditions. Das et al., (2020) also faced such spectral overlap complications in their UV-based Methotrexate analysis, which compromises specificity an issue that the current RP-HPLC technique well addresses [25].

By controlling CPPs in method development, the current study reduced out-of-specification results and thus increased method reliability. Hrichi et al., (2022) also showed comparable results in the case of cisplatin, where CPP control minimized errors in chromatographic analysis [26]. Furthermore, implementation of QbD and risk-based approaches is also consistent with Bas et al., (2021), who underlined the regulatory harmonization across the world through orderly validation protocols [27].

In summary, the current risk-based validation method maximized analytical performance, providing utmost sensitivity, precision, and regulation compliance. Contrast with the present literature underscore's reliability and reproducibility of the current RP-HPLC protocol compared to standard validation methods in important drug monitoring situations such as Methotrexate quantitation.

5. Conclusion

In conclusion, this study founds a strong and regulation-compliant analytical platform for determining Methotrexate via RP-HPLC and UV spectrophotometric methods in conformity with the principles of ICH Q2(R2) guidelines but assisted by risk-based thinking. Employing a synergistic blend of Quality by Design (QbD), Failure Mode and Effect Analysis (FMEA), and Box-Behnken Design (BBD), crucial method parameters were effectively identified and optimized, augmenting method reliability, precision, and robustness. The RP-HPLC analysis showed better performance with high linearity (R^2 > 0.999), high accuracy (98–102% recovery), and low %RSD (<2%), and was stable under forced degradation, which proved it to be a good stability-indicating method. Even though the UV method was simpler and quicker, it had lower specificity, especially under stress conditions. In total, the results underscore the importance of having systematized risk-based validation approaches to guarantee analytical method stability, regulatory compliance, and uniform quality control in the regular analysis of Methotrexate and other cancer chemotherapeutics.

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