Chinese Journal of Health Management
Volume 20 Issue 3, Year of Publication 2026, Page 511-524 | DoI-10.5281/zenodo.19661231

ISSN: 1674-0815

cjhmonline.com

DoI-10.564220/1674-0815

Chinese Journal of Health Management

Chinese Medical Association

Green Technology Approach: Optimized RP-HPLC Method for Accurate Quantification in 5FU employing AQBD approach

Pawar Sonali Baban [1*], Pratim Kumar Choudhury[1], Atul Arjun Baravkar[2], Preeti Rajabhau Meshram[3]

[1*]Pacific Academy of Higher Education and Research University, Udaipur, Rajasthan, India,313024
[1]Pacific Academy of Higher Education and Research University, Udaipur, Rajasthan, India,313024
[2]Agricultural Development Trust's School of Pharmacy and Research Centre, Baramati, Maharashtra, India 413115
[3]Department of Pharmaceutics, RJSPM College of Pharmacy, Pune, Maharashtra, India 411041
Corresponding Author Email id: **sonalipawar241216@gmail.com**

Article Information
Received: 22-12-2025
Revised: 13-01-2026
Accepted: 17-02-2026
Published: 26-03-2026

Keywords
5-Fluorouracil, RP-HPLC, Central Composite Design, AQbD, nanosuspension, green analytical chemistry.

ABSTRACT:
5-Fluorouracil (5-FU) is an extensively utilized antimetabolite chemotherapeutic agent. It requires precise and sensitive analytical approaches for its quantification in bulk drugs and advanced nano formulations. In the current study, a green, rapid, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and optimized using a Central Composite Design (CCD) under an Analytical Quality by Design (AQbD) framework to assess 5-FU in bulk and nanosuspension formulations. HPLC Chromatographic estimation was performed on a C18 column using a mobile phase of water: methanol (50:50 v/v), adjusted to pH 3.0 with orthophosphoric acid, at a flow rate of 1.0 mL/min in isocratic mode. Detection was carried out at 264 nm with an injection volume of 20 µL and a runtime of 6–10 minutes. The established technique displayed exceptional linearity over the concentration range of 10–100 µg/mL with a correlation coefficient ($r^2 > 0.999$). The technique was validated as per ICH Q2(R1) guidelines and confirmed satisfactory accuracy, precision, specificity, robustness, and sensitivity. The limits of detection (LOD) and quantification (LOQ) established the method's appropriateness for low-level quantification. CCD-based optimization enabled identification of critical method parameters inducing retention time, peak area, and tailing factor, confirming method robustness within the design space. Additionally, the use of environmentally sustainable solvents and reduced analysis time highlights the greenness of the method. The developed RP-HPLC method is modest, cost-effective, eco-friendly, and appropriate for routine quality control and analytical applications of 5-FU in bulk and nanosuspension systems.

INTRODUCTION:
5-Fluorouracil (5-FU) is described as a pyrimidine analog, as observed in Figure 1, which indicates the structure

of 5 -FU. It is one of the most commonly used antineoplastic agents. It is frequently employed in treating solid tumors, including colorectal, breast, and gastrointestinal cancers. Chemically, it is known as 5-fluoro-2,4-pyrimidinedione and works by blocking thymidylate synthase, which disrupts DNA synthesis and cell growth [1,2]. Comprising a narrow therapeutic window and speedy metabolism, a precise quantity of 5-FU is important to confirm precise dosing, therapeutic effectiveness, and safety.

5-FU is a hydrophilic drug with a log P of -0.89 to 0.98, and 12-15mg/mL water solubility. Its pka is 7.8 and is considered a weak acid and partially ionized near physiological pH, with low bioavailability and rapid systemic clearance. This manifested in the development of advanced drug delivery systems such as nanosuspensions to enhance their therapeutic presentation [3]. Nanosuspension-based formulations expand solubility, dissolution rate, and bioavailability, demanding consistent analytical methods for their assessment.

Numerous analytical practices, UV spectrophotometry, HPTLC, and HPLC, have been tested for the estimation of 5-FU in bulk and dosage forms. Nevertheless, the majority of these methods rely on conservative trial-and-error methods.

5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent with a narrow therapeutic index, rapid metabolism, and variable bioavailability, which necessitates precise and reliable analytical quantification to ensure therapeutic efficacy and patient safety. With the growing development of advanced drug delivery systems such as nanosuspensions, there is an increasing demand for highly sensitive, specific, and robust analytical methods capable of accurately estimating the drug in complex formulations. Conventional analytical techniques, including UV spectrophotometry and traditional HPLC methods, often rely on trial-and-error approaches, lack robustness, and involve the use of hazardous organic solvents. Moreover, regulatory guidelines such as ICH Q2(R1) emphasize the importance of validated, reproducible, and reliable analytical procedures. Therefore, there is a critical need to develop a green, rapid, RP-HPLC method that ensures accuracy, reproducibility, and environmental sustainability for routine analysis of 5-FU in both bulk and nanosuspension formulations [1–4].

The primary objective of the present study was to develop a simple, rapid, and environmentally friendly RP-HPLC method for the accurate quantification of 5-Fluorouracil in bulk drug and nanosuspension formulations Additionally, the method was intended to be validated in accordance with ICH Q2(R1) guidelines by evaluating parameters such as linearity, accuracy, precision, specificity, robustness, limit of detection, and limit of quantification. Another important objective was to establish a design space ensuring method reliability and reproducibility, while simultaneously incorporating green analytical chemistry principles to reduce solvent consumption and environmental impact.

The method showed novelty in the development of a green analytical method using eco-friendly solvent systems (methanol–water). Simultaneous quantification of 5-FU in both bulk drug and nanosuspension formulations. Establishment of a robust design space ensuring method reproducibility and regulatory compliance. Reduction in analysis time and solvent consumption, supporting sustainability. This integrated approach enhances analytical performance while aligning with modern regulatory and environmental expectations.

2. MATERIALS AND ANALYTICAL METHODS:

2.1 Materials and reagents

5-FU was obtained from Shubham Chemicals, Mumbai, India. Methanol (HPLC grade) and orthophosphoric acid were procured from standard chemical suppliers. Double-distilled water was used throughout the study. All reagents and solvents were of analytical or HPLC grade.

2.2 Instrumentation and Chromatographic Conditions

Subsequently, carrying out UV-visible spectrophotometric studies, the wavelength of maximum absorption for 5FU was found at 264 nm (Figure 2), indicating the maximum wavelength at which the drug absorbs. Consequently, this wavelength was additionally utilized as the operating wavelength for the subsequent HPLC studies. The mobile phase was designated depending on analysis time, sensitivity, and signal-to-noise ratio. Numerous trials for mobile phase compositions were taken, consisting of 0.1% acetic acid in methanol-water, plain methanol-water, acetonitrile-phosphate buffer pH 4 ± 0.2, and acetonitrile-methanol: water (50:25:25 % v/v). Finally, a mobile phase composed of methanol and water in a ratio of 50:50 % v/v (500 mL methanol and 500 mL water adjusted to pH 3.0 ± 0.05 with orthophosphoric acid (1% or 0.1 M) concentration) was used,

surveyed by using bath sonication for 15 minutes, for complete degassing, and filtered through a 0.45 µm membrane filter. The optimized mobile phase showed good reproducibility and system suitability. The chromatographic conditions included a flow rate of 1 mL/minute, detection wavelength 264nm, injection volume 2µL, Isocratic mode, and run time was kept for 6 to 10 minutes, respectively.

Chromatographic analysis was achieved by employing a Thermo-Ultimate 3000 High-performance liquid chromatography (HPLC) system manufactured by Thermofisher Scientific, installed with Chromaleon software. HPLC system equipped with a binary pump, UV detector, and manual injector with a 20 µL loop. Data acquisition and processing were carried out using suitable chromatographic software. Separation was achieved using a C18 column (250 × 4.6 mm, 5 µm). The mobile phase was filtered through a 0.45 µm membrane filter and degassed prior to use.

Figure 1. Structure of 5FU

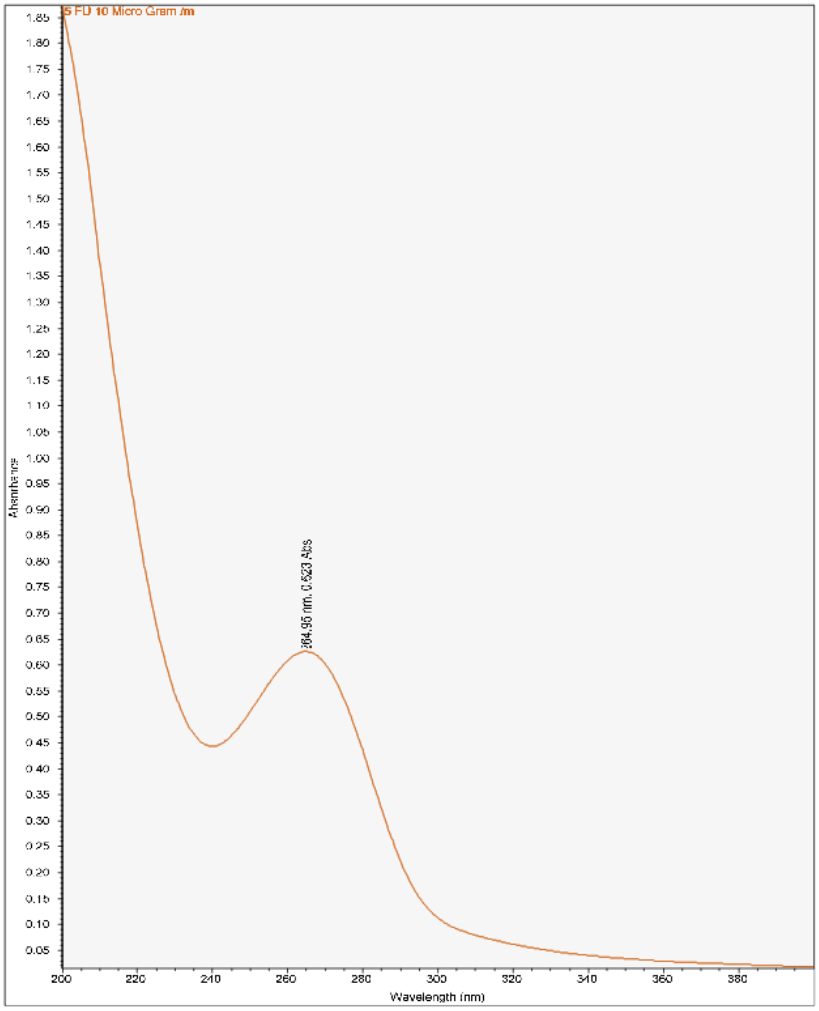


Figure 2. The maximum wavelength at which the 5FU absorbs

2.3 System suitability testing

The system suitability of 5-FU 2000 µg/mL was assessed by diluting a stock solution. The sample 20 µL was injected six times, and many constraints were quantified, including theoretical plates, retention time, tailing factor/asymmetry, and %RSD. In accordance with standard ICH guidelines, the % RSD should not be more than

2%, the asymmetry factor should be below 2%, and the theoretical plates (TP) (measures column's efficiency) should be more than 2000. Reduced TP specified non-reproducibility in peak areas.

2.4 Preparation of Standard Stock Solution and Working Standards

An exactly weighed quantity of 5-FU (200 mg) was transferred into a 100 mL volumetric flask and dissolved in the methanol: water (50:50) v/v. The volume was adjusted to obtain a stock solution of 2000 µg/mL.

2.5 Preparation of Working Solutions

Working standard solutions in the concentration range of 10–100 µg/mL were prepared by suitable dilution of the stock solution with the methanol: water (50:50) v/v. All solutions were filtered through a 0.45 µm membrane filter before injection.

3. Method Validation

The developed RP-HPLC method was validated as per ICH Q2(R1) guidelines for the following parameters, including linearity, accuracy, precision, specificity, robustness, LOD, and LOQ, respectively.

3.1 Linearity and range

The linearity studies were directed to certify that the concentrations of the sample solutions used in the study formed constant and precise consequences within the detailed range. Beer-Lambert law, principal represent that the peak area of the drug should be directly proportional to the concentration. For employing the linearity test, 10-100 µg/mL concentration of 5-FU solutions were made from standard stock solutions and diluted with methanol: water (50:50 v/v). The study samples were inserted into the HPLC system, and peak areas were documented accordingly. The attained peak areas were designed in contradiction to concentration, and the correlation coefficient (r^2) and calibration equation with slope were designed.

3.2 Accuracy (% recovery)

The accuracy of the technique was validated, retaining standard addition and recovery tests. The studies elaborate on the addition of three different concentration levels (50%, 100%, and 150% of the target concentration of 10 µg/mL) in triplicate. The percentage recovery and relative standard deviation (%RSD) were designed, yielding values below 2%. Peak areas from the samples were compared to those of a 5FU standard solution, and the percentage recovery of the total drug was determined using a specific formula. The mean percentage recovery±standard deviation (S.D.) was stated for each concentration level. An acceptance criterion for recovery was established between 98% to 102%. The percent recovery was considered utilizing the formula:
(Found concentration/Total concentration) x 100[5].

3.3 Specificity

Specificity was directed to certify that the technique precisely recognizes the target analyte. The calculation can be done by associating the data of the drug solution before spiking and after spiking the drug solution, which discovered that there should not be the presence of significant interference of blank with the recovery of 5- FU, concluding that the method was specific.

3.4 Precision

Precision supports the calculation of the degree of agreement among individual measurements. To calculate intra-day and inter-day precision, three samples encompassing concentrations falling within the low, medium, and high levels of the calibration range were subjected to triplicate analysis. Intra-day precision involved analyzing solutions of 5- FU at concentrations of 10, 50, and 100 µg/mL three times within the same day. During inter-day precision, the same concentrations of the drug were analyzed over three consecutive days. The subsequent area values were applied to compute the %Relative Standard Deviation (%RSD), which ideally should not exceed 2% for the drug[8].

3.5 Limit of detection (LOD) and Limit of quantification (LOQ)

The standard deviation of the response method was selected among the three methodologies available. LOD and LOQ were established based on the calibration curve method, employing the residual standard deviation technique. For 5-FU, this establishment was directed through a standard calibration curve applying data analysis in Excel, obeying the experimental parameters. The sensitivity quantity, which involves achieving a signal-to-noise ratio of 3: 1 for LOD and 10: 1 for LOQ, was calculated by dividing the K* standard deviation of the peak

response area by the slope, where k equals 3.3 and 10 for LOD and LOQ, respectively. Sensitivity, fixed as the signal-to-noise ratio, should be maintained at levels where the calculated %RSD does not exceed 10%[8].

3.6 Robustness using the conventional and AQBD approach

The conventional methods utilized (OFAT), i.e., one factor at a time, were found to be laborious and untrustworthy after comparing with the AQbD approach. It assesses only the interaction between variables and is unable to deliver optimized robustness. It is an experimental method in which the presentation of the method was measured during validation; the excellence of the procedure is contingent only on faultless validation. This method did not use any regulatory guidelines and does not have any space for further enhancement. On the other hand, AQbD, which is comprised of a prearranged goal, sets an analytical target outline by concentrating on presentation. It can assess variables and statement effects systematically. Hence, for optimized robustness evaluation, the AQbD Approach was employed. Thus, the Analytical Quality by Design (AQbD) approach can meaningfully improve the robustness of analytical methods. AQbD highlights a methodical outline that involves risk assessment and design of experiments, leading to more dependable and well-organized method development. AQbD creates a method operable design space (MODS), which permits the documentation of optimal conditions while minimizing variability. The AQbD method recognizes initial risk valuation and resolves possible issues, making the developed method more reliable.

Firstly, a conventional method was employed to measure robustness by purposely varying the mobile phase composition and flow rate, and then monitoring the method's performance through system suitability tests. Variations were applied within precise tolerances: ±0.1 mL/min for flow rate, ±0.2 for pH, and ±2% for mobile phase composition. For instance, with 5-FU, three samples of a 100 µg/mL solution were injected at flow rates of 0.9, 1.0, and 1.1 mL/min, using a mobile phase of methanol: water at pH 3 ±0.05, with varying ratios of 53: 47, 50: 50 and 47: 53 (v/v) employing orthophosphoric acid. The impact of these adjustments on parameters like retention time and peak areas was assessed in terms of %R.S.D. values.

In contrast, the AQbD approach employed a Central Composite Design with three levels, incorporating 6 axial points, 6 center points, and 8 factorial points, totaling 20 batches as suggested by Design Expert software version 13. Factors such as pH, %organic content, and flow rate were considered, with target responses being retention time (R.T), peak area (P.A), theoretical plate (T.P), and tailing factor (T.F). Applying Design Expert software, the percentage influence of each factor was predicted and surveyed by ANOVA statistical analysis. Moreover, perturbation plots, 3D response surface plots, contour plots, and overlay plots were generated for graphical representation and optimization of the method.

4. RESULTS AND DISCUSSION:

4.1 Development of the HPLC method for 5FU

For performing analysis, an appropriate wavelength selection is needed. The optimized wavelength was verified by a UV spectrophotometer (Shimadzu UV-1800) at a wavelength of 264 nm. The mobile phase optimized for the study was 50:50 v/v water: methanol. Hence, this mobile phase was selected for further use. The selection of a solvent for the extraction of the drug was also required for 5FU recovery. Thus, plain methanol, acetonitrile, and mobile phase, and a 50:50 ratio of water and methanol mixture were employed for the best solvent selection. Three different solutions of 5FU (1000 µg/mL) were made in methanol, acetonitrile, and the mobile phase, and a 50:50 ratio of water and methanol mixture. R.T, % R.S.D, and recovery were calculated from the calibration equation using a standard calibration curve in respective solvents. The best recovery was observed with a methanol and water mixture as indicated in Table 1.

Table 1: Trials were conducted for the recovery of 5FU (100 µg/mL) in different solvents for the selection of the extracting medium

Extracting medium	Mean peak area ± S.D(n=3)	R. T	% RSD	% Recovery
Methanol	48710.32 ± 123.21	2.912	1.04	94.52 ± 1.55
Acetonitrile	23568.43 ± 236.98	2.523	1.16	74.65 ± 2.94
Mobile Phase	20369.38 ± 213.67	2.212	1.45	60.34 ± 1.98
Acetone	19854.23 ± 12532	3.614	2.85	55.35 ± 2.38
Water: Methanol (50:50)	48810.56 ± 104.21	3.084	0.65	98.36 ± 2.38

R.T- Retention Time, % RSD- (Relative standard deviation), ACN- Acetonitrile

The optimized RP-HPLC method developed using a green analytical approach demonstrated excellent

chromatographic performance for the quantification of 5-Fluorouracil (5-FU). The representative chromatogram (as shown in Figure 3) exhibited a sharp, well-defined, and symmetrical peak with a retention time of approximately 3.084 minutes, indicating efficient separation under the selected conditions. The absence of additional interfering peaks confirms the specificity of the method, suggesting that excipients or formulation components in the nanosuspension do not interfere with the detection of 5-FU.

The peak shape was found to be symmetrical with minimal tailing, indicating proper interaction between the analyte and the C18 stationary phase at acidic pH (pH 3.0). The high peak intensity (~5000 mAU) reflects good detector response and sensitivity of the method. The short retention time further supports the rapid analysis capability**,** making the method suitable for routine quality control.

Baseline stability was observed throughout the run, with no significant noise or drift, confirming proper mobile phase compatibility and system performance. The system suitability parameters, such as theoretical plates and tailing factor, were within acceptable limits, demonstrating high column efficiency and reproducibility.

Overall, the chromatographic profile confirms that the developed method is precise, selective, sensitive, and robust**,** and is highly suitable for the accurate quantification of 5-FU in bulk as well as nanosuspension formulations under green analytical conditions.

4.2 System Suitability Parameters

The system suitability results for the developed RP-HPLC method of 5-FU demonstrated excellent chromatographic performance and reproducibility. The retention time was found to be 3.084 ± 0.012 min**,** indicating consistent and rapid elution of the analyte under the optimized conditions. The peak area showed a mean value of 499822 ± 35 mAU**,** with a %RSD of 0.70%**,** which is well within the acceptable limit of ≤ 2%, confirming the precision and repeatability of the system. The column efficiency was satisfactory, as evidenced by the theoretical plate count of 3560 ± 45**,** exceeding the minimum requirement of 2000. Additionally, the tailing factor was observed to be 1.21 ± 0.03**,** indicating a symmetrical peak and proper interaction between the analyte and the stationary phase. No additional peaks were detected, confirming the specificity of the method with a single, well-resolved peak. Overall, all system suitability parameters complied with standard acceptance criteria, demonstrating that the chromatographic system is reliable, precise, and suitable for routine quantitative analysis of 5-FU (Table 2, represented System Suitability Results of 5-FU (n = 6 injections). The system suitability results confirm that the chromatographic system is performing adequately. The low %RSD (< 2%), high theoretical plates (>2000), and acceptable tailing factor (< 2) indicate that the developed method is reliable, reproducible, and suitable for analysis.

Table 2. System Suitability Results 2000 µg/mL of **5-FU (n = 6 injections)**

Parameter	**Result (Mean ± SD)**	**Acceptance Criteria**	**Interpretation**
Retention Time (min)	3.084 ± 0.012	—	Consistent elution
Run time	10 minutes		
Injection volume	20µL		
Mean peak area (mAU)	499822 ± 35	%RSD < 2%	Acceptable
%RSD (Peak Area)	0.70%	≤ 2%	Excellent precision
Theoretical Plates (N)	3560.2 ± 45	> 2000	Good column efficiency
Tailing Factor	0.96 ± 0.03	< 2	Symmetrical peak
Resolution	—	—	Single peak observed

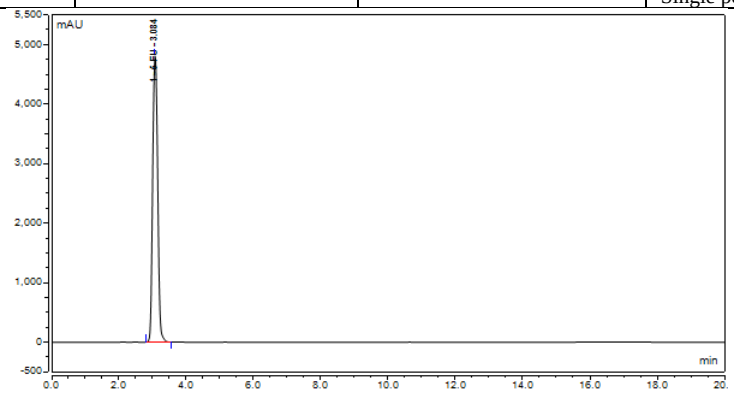


Figure 3. A Typical peak of 5FU

5. Method Validation Results (ICH Q2(R1))

The analytical method was thoroughly verified in accordance with the validation requirements outlined in the ICH guidelines, with additional reference to established industry guidance on analytical procedures and method validation. This structured validation approach ensured that the method met regulatory expectations for reliability, accuracy, and reproducibility, as demonstrated through the associated Figures and Tables presented in the study.

5.1 Linearity, sensitivity, and range

Linearity of the method was evaluated by preparing 5FU solutions over a concentration range of 10–100 µg/mL. The calibration data showed a linear relationship between concentration and response, with a regression equation of $Y = 473.73x + 1605$ and a correlation coefficient (r^2) of 0.9957, indicating excellent linearity. The detailed linearity results are presented in Table 3**,** while the corresponding calibration curve is illustrated in Figure 4**.** Based on these findings, the developed method demonstrated good linear behaviour across the studied concentration range and was found to comply with Lambert's law.

Table 3. Standard calibration values of 5FU using diluent (Methanol: water, 50:50).

Concentrations (µg/mL)	Area
10	6297 ± 1.08
20	12109 ± 2.98
30	18056± 5.12
40	20256± 4.02
50	24498 ± 4.02
60	30512 ± 3.52
70	35689 ± 6.12
80	40298 ± 5.12
90	42587 ± 1.02
100	48810 ± 1.09

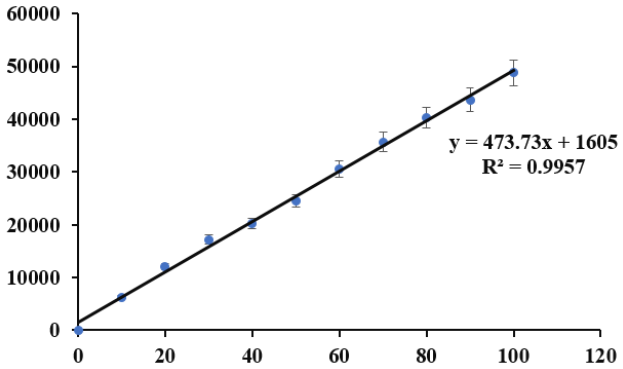


Figure 4. Linearity of 5FU in water

5.2 Accuracy/ recovery studies

Accuracy was assessed by spiking known amounts of the analyte into the sample and comparing the measured concentration with the expected value. The accuracy of the method was evaluated through recovery studies of 5FU at three concentration levels—80%, 100%, and 120%—with each level analyzed in triplicate. The results demonstrated that the method was accurate, with mean percentage recoveries ranging from 99.08 ± 0.52% to 100.38 ± 0.39%, as summarized in Table 4. These recovery values were within the acceptable limits of 100 ± 2%, confirming the accuracy of the developed method.

Table 4. Accuracy/Recovery Values of 5FU

Level Concentration %	Target concentration (µg/mL)	Spiked Conc. (µg/mL)	Total conc. (µg/mL)	Area found	Found conc. (µg/mL)	Percent recovery	Mean percent recovery
80	30	24	54	25945 ± 0.90	53.91 ± 0.92	99.83 ± 0.92	
80	30	24	54	26589 ± 0.58	53.78 ± 0.90	99.59 ± 0.24	
80	30	24	54	27482 ± 0.22	53.62 ± 0.10	99.30 ± 0.12	99.57± 0.54
100	30	30	60	30512 ± 0.23	59.85 ± 0.21	99.75 ± 0.19	
100	30	30	60	31256 ± 0.21	59.45 ± 0.18	99.08 ± 0.52	99.68± 0.39

100	30	30	60	31254 ± 0.22	60.12 ± 0.69	100.20 ± 0.46	
120	30	36	66	50854 ± 0.22	65.88 ± 0.78	99.82± 0.52	99.89± 0.48
120	30	36	66	51245 ± 0.22	66.25 ± 0.65	100.38 ± 0.39	
120	30	36	66	51421 ± 0.22	65.65± 0.8	99.47± 0.10	

5.3 Precision (Repeatability studies)

Intra-day precision (repeatability) of the developed method was evaluated by analyzing three replicate injections at three different concentration levels (10, 20, and 30 µg/mL) within the same day, conducted during the morning, afternoon, and evening sessions. Inter-day precision was assessed by repeating the same analytical procedure over three consecutive days. The precision results are summarized in Table 5.

The intra-day precision showed % RSD values ranging from 0.254 to 0.658, while the inter-day precision exhibited % RSD values between 0.318 and 0.968. In all cases, the % RSD values were well below 2 for 5FU, confirming that the method demonstrates excellent precision and reproducibility.

Table 5. Intra-day and Inter-day Precision reports of 5FU

Concentration assayed (µg/mL) (n= 3)	**Intra-day (repeatability)**		**Inter-day (Intermediate)**	
	Average area		**Average area**	
	Mean	**% RSD**	**Mean**	**% RSD**
10	314261	0.658	312971	0.968
20	694467	0.368	692277	0.394
30	985963	0.254	987296	0.318

5.4 Limit of Detection and Limit of Quantification (LOD & LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) for 5FU were determined to be 0.192 µg/mL and 0.852 µg/mL, respectively. These values indicate that the developed method possesses good sensitivity for both detection and accurate quantification of 5FU at low concentration levels. The calculated LOD and LOQ results are presented in Table 6**.**

Table 6. LOD and LOQ Values of 5FU

Parameter	**Value obtained**
LOD(µg/mL)	0.192
LOQ(µg/mL)	0.852

5.5 Robustness by the conventional and AQBD approach

During method validation, key HPLC chromatographic parameters such as flow rate, mobile phase composition, and pH were intentionally varied to evaluate the robustness of the method. Under these deliberately modified conditions, all analytical parameters were satisfactorily assessed, and the chromatographic conditions were optimized to ensure effective separation of the analytes. Critical responses, including peak area and retention time, remained consistent despite controlled variations in flow rate, mobile phase composition, and mobile phase pH. The acceptable variation limits were set at ± 0.1 mL/min for flow rate, ± 0.2 units for pH of the mobile phase, and ± 2% for mobile phase composition.

The % RSD values obtained after introducing these minor, carefully controlled changes confirmed that the method is robust for the intended analysis. The robustness results obtained using the conventional approach are presented in Table 7.

Table 7. The Standards for robustness in method development

Parameter	**Method condition**	**% RSD of peak area**	**% RSD of retention time**
Flow rate (mL/min) (±0.1)	0.9	0.21%	0.20%
	1.0	0.34%	0.18%
	1.1	0.51%	1.00%
Composition of Mobile phase (v/v) (± 2%)	Water: Methanol, pH 3.5 ± 0.05 in the ratio of 53:47 v/v	0.15	0.30%
	Water: Methanol, pH 3.5 ± 0.05 in the ratio of 50:50 v/v	0.34%	0.25%
	Water: Methanol, pH 3.5 ± 0.05 in the ratio of 47:53 v/v	0.54%	0.52%
pH of mobile phase (±0.2)	Water: Methanol, pH 3.2 ± 0.05 in the ratio of 50:50 v/v	0.92%	0.21%

	Water: Methanol, pH 3 ± 0.05 in the ratio of 50:50 v/v	0.20%	0.56%
	Water: Methanol, pH 3.4 ± 0.05 in the ratio of 50:50 v/v	0.52%	0.93%

While conducting validation studies, chromatographic constraints such as pH, mobile phase composition, and flow rate were intentionally adjusted. During studies, all analyses were sufficiently committed, and the order of elution continued unaffected. This means that the chromatographic conditions were optimized to attain the anticipated separation of the analytes. The selected factors, including peak area and retention time, endured genuine after creating variations in the pH, mobile phase composition, and flow rate. Flow rate should be ±0.1, pH of mobile phase ±0.2, and composition of mobile phase ±2% acceptable. The %RSD values obtained after making small deliberate changes in the method indicate that the method is robust for the intended purpose, as manifest in Table 7. In contrast, the Novel AQbD approach, CCD design was employed to perform a robustness study, which was indicated in Table 8. Factors or critical method parameters (CMPs) were selected, such as pH, %organic, and flow rate, at three levels. Four Critical Quality Attributes (CQAs) ie. responses taken were retention time, area, tailing factor, and number of theoretical plates. For the study of the application of Analysis of Variance (ANOVA) to all response variables to examine the significance of the model, which showed that all the responses achieved had significant differences in their values. Equations obtained from the model were:

Retention time Y1 = +3.13+0.0513 A - 0.0220 B – 0.0636C – 0.0875AB – 0.0375AC +0.0375BC (1)
PeakArea(Y2) = +32603.3 +1332.47A-1579.34B-1531.48C-2899.62AB-2559.87AC+2443.88BC ...(2)
TP(Y3) =+3722.40+333.16A-270.98B-160.31C-248.00AB-551.50AC+187.00BC ....(3)
TF=+1.45-0.0124A-0.1803B-0.0234C-0.3450AB-0.1950AC+0.0100BC .....(4)

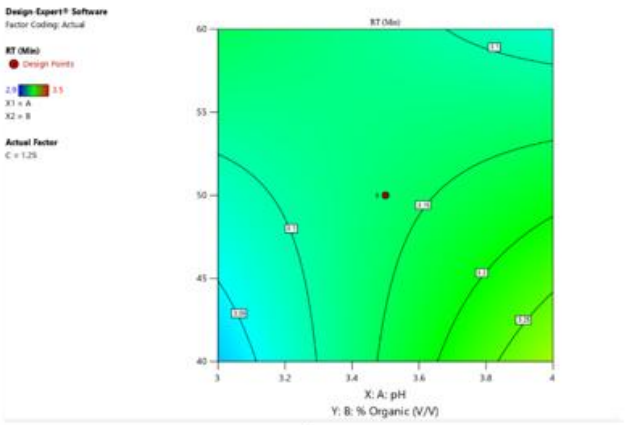
From the Equations, a positive sign designates a synergistic effect, a negative sign indicates an antagonistic effect in the polynomial equation. The responses Y1, Y2, Y3, and Y4 indicated that predicted values for all the factors are below a satisfactory value. The 2FI model was suggested by software for all CQA, and P-values (less than 0.05) were found to be significant for all responses.

Graphical interpretation in the form of 3-D response surface plots and contour plots, showed the correlation of the effect of factors on the response retention time, area, tailing factor, and theoretical plates of 5FU analysis. The model was evaluated for the effect of individual factors on the responses in the form of 3-D response surface plots and contour plots. In Design Expert software, 3D plots and contour plots serve as powerful visualization tools for analyzing experimental data and understanding the relationships between variables in a design space. The main purpose of a 3D Plot is to visualize response surfaces, which depict the relationship between multiple independent variables (factors) and a response variable (output). By plotting the response variable on the z-axis and two factors on the x and y axes, you can see how the response changes as the factors vary. 3D plots can help identify regions of the design space where the response variable is optimized or minimized. This is particularly useful in optimization studies where the goal is to find the best combination for visualizing and interpreting experimental data, identifying trends, and making informed decisions in the design and optimization of processes or systems. All responses in 3D and contour plots were indicated in Figure 5(A-H). In the context of Design Expert software, perturbation generally refers to the systematic variation of factors or variables within a design matrix. This variation helps in understanding the system's sensitivity or process to changes in these factors. During the perturbation of a design in Design Expert software version 13, the design matrix was modified by changing the levels or values of factors to observe how the response variables behave under these altered conditions, which allows the analysis of the robustness of the design and make informed decisions about optimizing your process or system indicated in (Figure 6 (A-D) i.e. (R.T, Area, T.P, and T.F) are the responses denoted by Y1, Y2, Y3, and Y4, whereas pH (A), % organic content (B), or f low rate (C) is the actual factors). Perturbation quantifies the number of deviations from its central point, which is (reference). During the perturbation process in the Design Expert software version13, alterations are made to one factor at a time, which can be pH (A), % organic content (B), or flow rate (C)-from the reference point, which is the design space's center point, and responses Y1, Y2, Y3, and Y4 are observed. Perturbation plots indicate the independent effect of each factor by performing a minute change in one factor while keeping the others constant. These plots provide insights into robustness. In a perturbation plot, a relatively flat line for a factor (A, B, or C) indicates that the response variable is not significantly influenced by changes in that factor. Conversely, a steep slope indicates a high sensitivity of the response variable to changes in that factor. A curved line suggests a nonlinear relationship between the factor and the response variable. For instance, the perturbation plot of retention time showed significant deviation from the

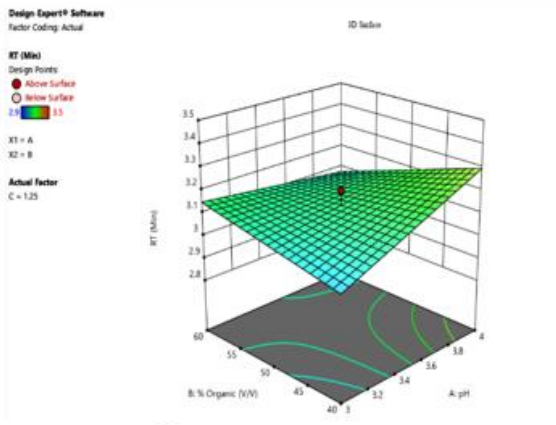
center point, with a curvature line indicating a nonlinear relationship between the factors (A, B, C) and the response variable retention time (R.T). In contrast, the perturbation plot of the area displayed less deviation from the reference point and a steep slope, suggesting that the area is highly sensitive to changes in that factor. The perturbation plot of the theoretical plate (T.P) showed deep curvature, indicating more deviation from the reference point and a nonlinear relationship between the factor and the response variable. Meanwhile, the tailing factor plot (T.F) exhibited minimal deviation from the center point, with flat lines suggesting that the response variable is highly sensitive to changes in that factor. The multi-dimensional combination of input variables and process parameters assures quality, which is called the design space, as indicated in Figure 7, which tells us the desirability plot for the optimized solution. The overlay plot in Figure 8 displays the design space. The yellow region shows that, by varying the experimental variables in this region, the method remains robust.

Table 8. Central composite design for robustness testing using factors and obtained responses

Run	Factor 1 A: pH	Factor 2 B: Mobile phase composition(%)	Factor 3 Flow rate mL/min	Response 1 R.T (minutes)	Response 2 Peak area (mAU)	Response 3 TP	Response 4 TF
1	4	60	1.5	3	30512	3565	0.96
2	4.3	50	1.2	3.1	31543	3560	1
3	3.5	50	1.2	3.2	32514	3652	1.1
4	3	60	1	3.2	31023	4023	1.2
5	3.5	40	1.5	2.9	31203	4125	1.5
6	3.5	50	1.2	3.1	35236	4152	1.9
7	3.5	50	12	3	32145	4125	1.8
8	3.5	33	1.2	3.2	31256	3986	1.5
9	3.5	50	1.6	3.1	31256	3256	1.6
10	3.5	50	1.2	3.1	32145	3545	1.4
11	3.5	66	1.2	3.2	30256	3674	0.94
12	3	60	1.5	3.1	31548	3365	1.62
13	3.5	50	1.2	3.2	31256	3354	1
14	2.6	50	1.2	3.1	31456	3145	1.6
15	3	60	1	3.1	32560	3289	1.5
16	4	40	1.5	3.2	31025	3645	1.8
17	3.5	50	1.2	3.1	31568	3125	1.5
18	3.5	50	0.8	3.2	30256	3214	1.6
19	3	40	1	3.1	31250	3125	1
20	4	40	1	3.5	52052	6523	2.5

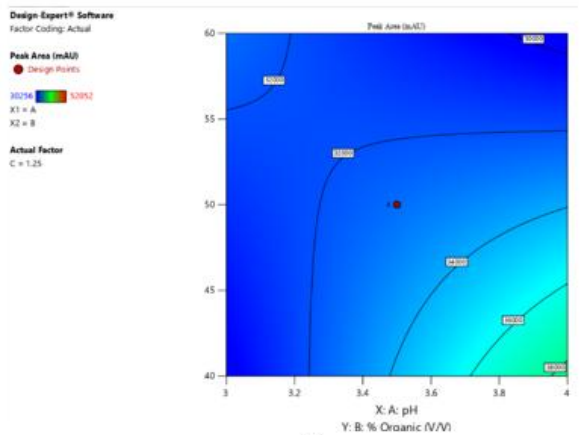


A

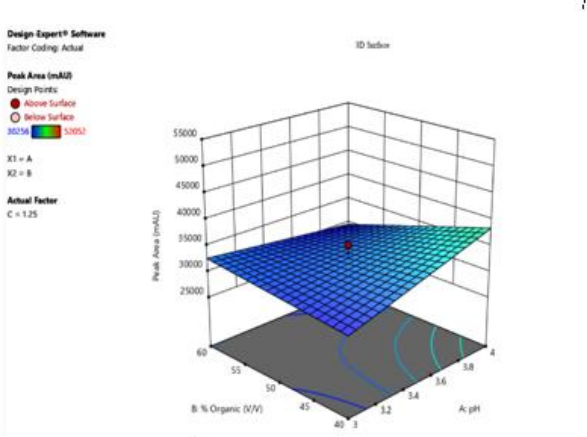


B

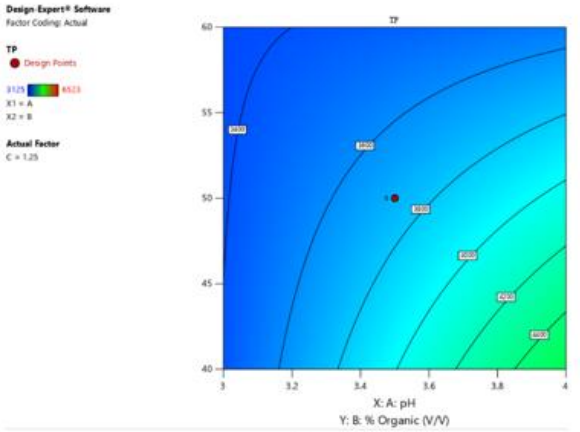
Chinese Journal of Health Management
Volume 20 Issue 3, Year of Publication 2026, Page 511-524 | DoI-10.5281/zenodo.19661231



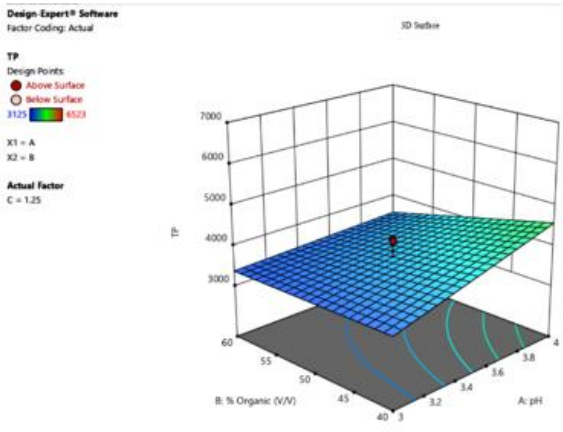
C



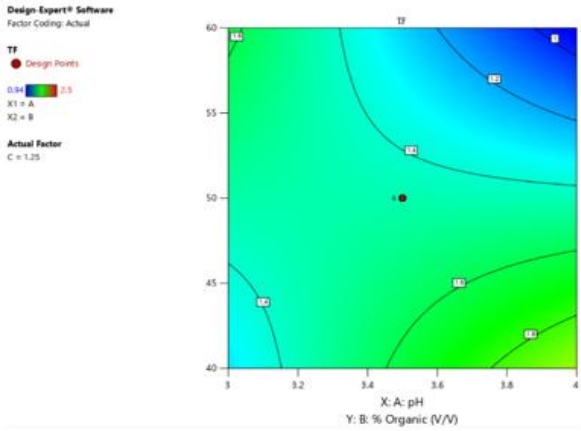
D



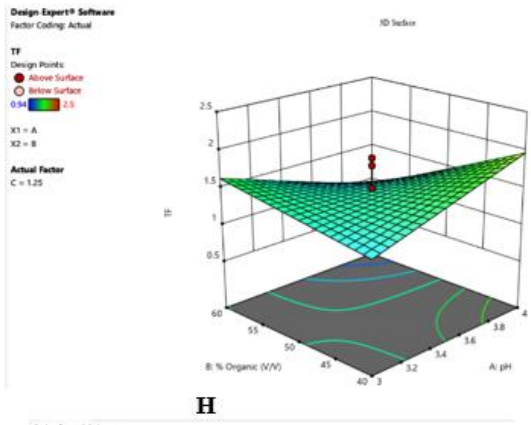
E



F



G



H

Chinese Journal of Health Management
Volume 20 Issue 3, Year of Publication 2026, Page 511-524 | DoI-10.5281/zenodo.19661231

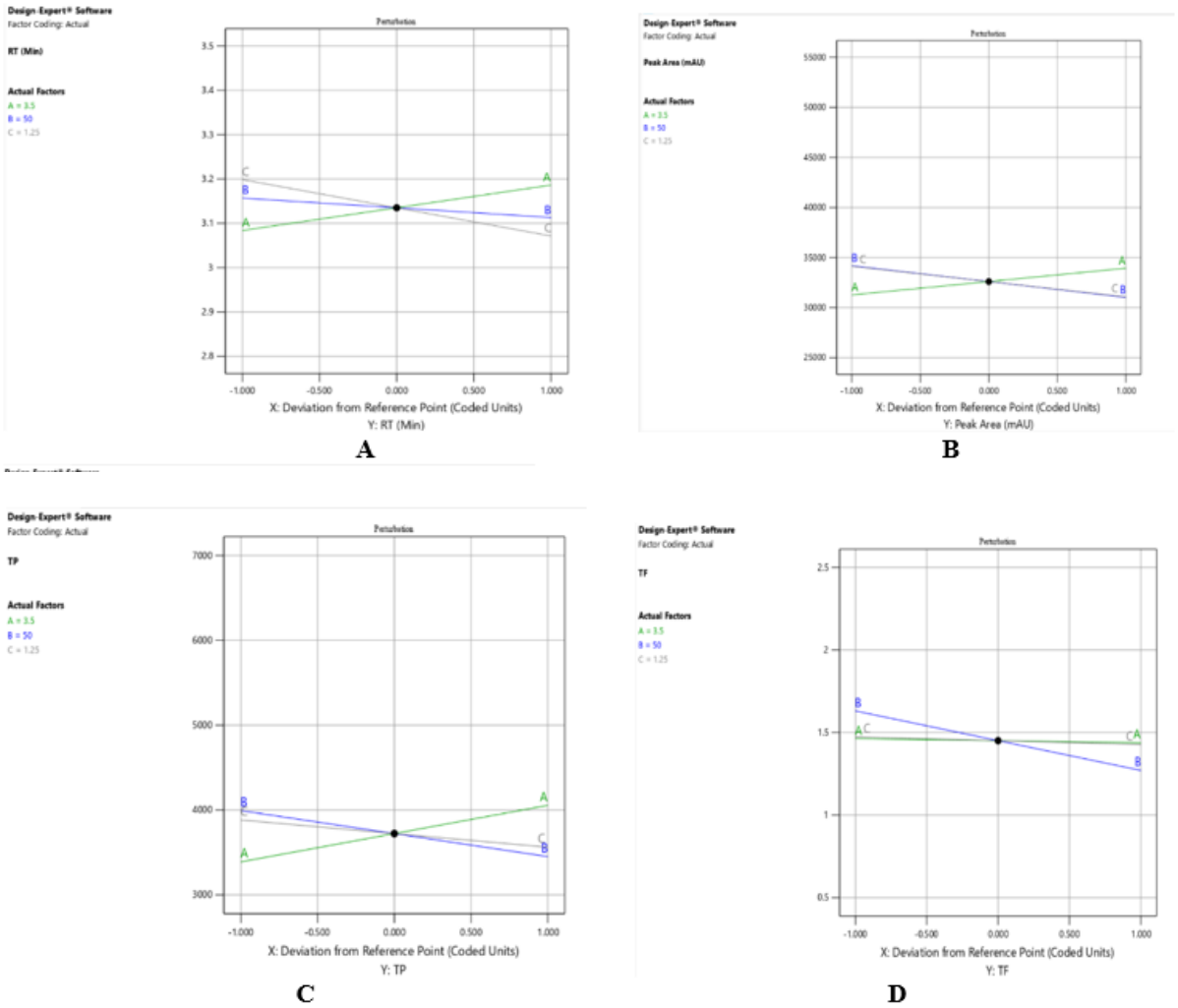


Figure 7. Perturbation plots (A-D) showing the effect of factors on responses (RT, Area, TP, & TF)

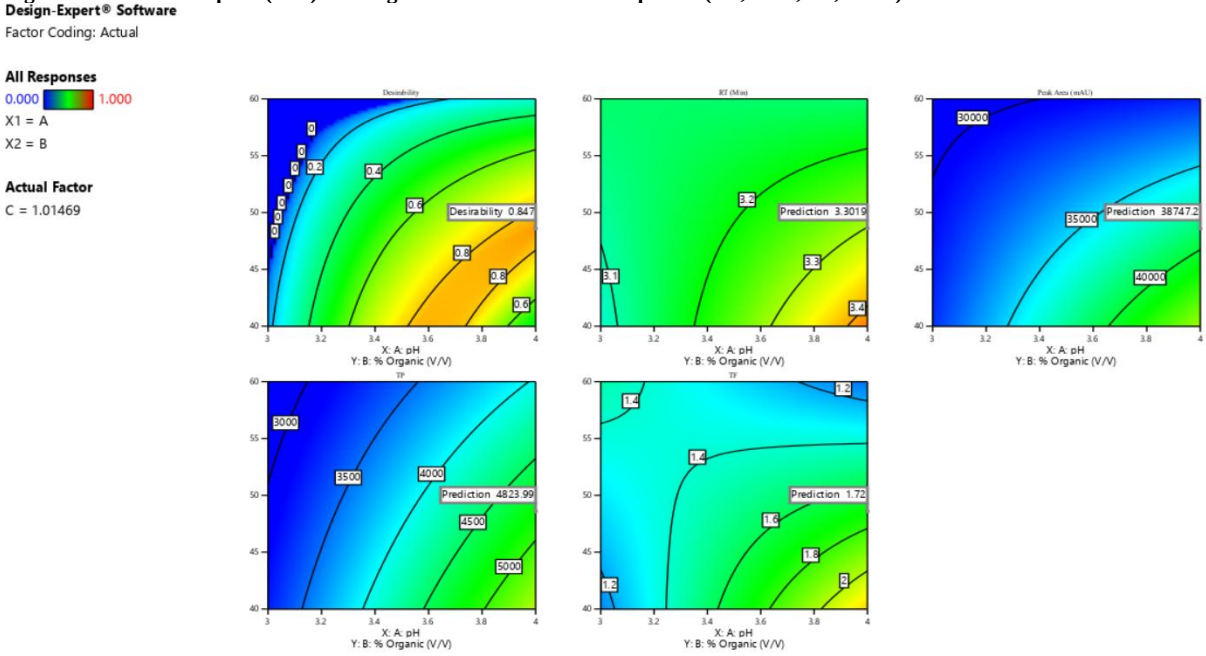


Figure 7. The desirability of contour plots

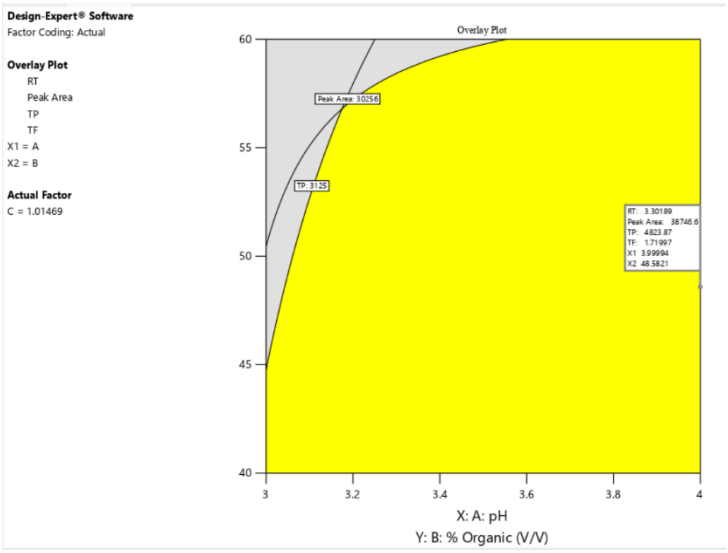


Figure 8. Overlay plot

6. DISCUSSION

The developed RP-HPLC method exhibited excellent chromatographic performance for the quantification of 5-Fluorouracil. The method produced a sharp, well-defined, and symmetrical peak with a retention time of approximately 3.084 minutes, indicating efficient separation and rapid analysis. The absence of interfering peaks confirmed the specificity of the method, demonstrating that formulation excipients did not affect drug detection. The method showed excellent linearity over the concentration range of 10–100 µg/mL with a high correlation coefficient, indicating its suitability for quantitative analysis. The low values of limit of detection and limit of quantification reflected the high sensitivity of the method. Accuracy studies demonstrated recovery within acceptable limits (98–102%), while precision studies showed %RSD values less than 2%, confirming the reliability and reproducibility of the method. Robustness testing revealed that small deliberate variations in chromatographic conditions did not significantly affect analytical performance, indicating the stability of the method. The application of Central Composite Design enabled systematic optimization of critical method parameters and establishment of a design space, reducing variability and enhancing method understanding. Furthermore, the use of a methanol–water solvent system and reduced run time highlights the eco-friendly nature of the method, aligning with green analytical chemistry principles [5–8].

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the analysis, interpretation, and drafting of the manuscript.

LIST OF ABBREVIATIONS

RT = Retention time
Conc. = Concentration
RSD = Relative Standard Deviation
F.R = Flow rate
% = Percentage
min = Minutes
mL = milliliter
v/v = Volume/Volume
ng = Nanogram
µL = Microlitre

AVAILABILITY OF DATA AND MATERIAL

The data that support the findings of this study are available from the corresponding author upon request.

CONFLICTS OF INTERESTS

No conflicts of interest

7. CONCLUSION:

In conclusion, a green, rapid, and robust RP-HPLC method for the quantification of 5-Fluorouracil was successfully developed and validated. The method demonstrated excellent linearity, accuracy, precision, sensitivity, and robustness in accordance with ICH Q2(R1) guidelines. Additionally, the incorporation of environmentally friendly solvents and reduced analysis time supports the principles of green analytical chemistry. The developed method is simple, cost-effective, and highly suitable for routine quality control and analytical applications of 5-FU in both bulk drug and nanosuspension formulations.

REFERENCES

1. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3:330–338.
2. Peters GJ. Mechanisms of action of 5-fluorouracil. Cancer Treat Rev. 1991; 18:215–230.
3. Patravale VB, Date AA, Kulkarni RM. Nanosuspensions: a promising drug delivery strategy. J Pharm Pharmacol. 2004; 56:827–840.
4. ICH Q2(R1). Validation of Analytical Procedures: Text and Methodology. 2005.
5. Snyder LR, Kirkland JJ, Dolan JW. Introduction to Modern Liquid Chromatography. 3rd ed. Wiley, 2010.
6. Swartz ME, Krull IS. Analytical Method Development and Validation. CRC Press; 2012.
7. Montgomery DC. Design and Analysis of Experiments. Wiley; 2017.
8. Meshram P, Ranpise N. Green AQbD-based HPLC method development. Anal Chem Lett. 2024; 14:909–934.
9. Chen, H., Wang, R., McElderry, J.D. (2023). Discriminative Dissolution Method Development Through an AQbD Approach. AAPS Pharm Sci Tech. 8: 255.
10. Santhanam, M.K., Nagarajan, N.C., Ponraj, P.B., Mohd-Hilurudeen, M.S. (2023). A Complete Roadmap of Analytical Quality by Design in Various Analytical Techniques. Current Pharma Anal. 3: 184-215.
11. Das, P., Maity, A., Analytical quality by design (AQbD). (2017). A new horizon for robust analytics in pharmaceutical processes and automation. International Journal of Pharmaceutical and Drug Analysis. 8: 324-37.
12. Mishra, S.M., Rohera, B.D. (2017). An integrated, quality by design (QbD) approach for design, development, and optimization of orally disintegrating tablet formulation of carbamazepine. Pharma development and tech. 7: 889-903.