IJMPR, 2026, 10(1), 01-08 ISSN: 2319-5878

INTERNATIONAL JOURNAL OF MODERN PHARMACEUTICAL RESEARCH

www.ijmpronline.com

Review Article

Impact Factor: 6.669

Coden USA: IJMPR3

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF 5-FLUOROURACIL AND BETANIN IN BULK AND SUSTAINED-RELEASE FORMULATIONS

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Article Received on: 26/11/2025 **Article Revised on: 16/12/2025** **Article Published on: 01/01/2026**

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https://doi.org/10.5281/zenodo.18107014

How to cite this Article: *1Drx. Krishnakant Ajit Choure, 2Dr. Sushama Vaishnav, 3Dr. Sachin S Bhusari, 4Dr. Pravin S Wakte. (2026). Development And Validation Of A Stability-Indicating Rp-Hplc Method For Simultaneous Estimation Of 5-Fluorouracil And Betanin In Bulk And Sustained-Release Formulations. International Journal of Modern Pharmaceutical Research, 10(1), 01–08.

ABSTRACT

This study focuses on the development and Analytical Quality by Design (AQbD)–driven optimization of a stability-indicating reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of 5-Fluorouracil (5-FU) and Betanin in both bulk drug substances and sustained-release pharmaceutical formulations. A comprehensive risk assessment using Ishikawa fishbone analysis identified critical method parameters, which were systematically optimized using a 3^2 factorial Design of Experiments (DoE) approach. Critical method variables including mobile phase composition (methanol:acetonitrile ratio), buffer pH, and flow rate were investigated to establish their influence on chromatographic performance parameters such as resolution, tailing factor, and retention time. Response Surface Methodology (RSM) facilitated the development of predictive mathematical models and establishment of a Method Operable Design Region (MODR) ensuring robust analytical performance. Comprehensive forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic stress conditions confirmed the method's stability-indicating capability with adequate resolution between parent compounds and their degradation products. The developed method was rigorously validated according to ICH Q2(R2) guidelines, demonstrating excellent linearity ($R^2 > 0.999$), accuracy (recovery 98.5-100.5%), precision (RSD < 1.0%), specificity, robustness, and appropriate sensitivity (LOQ < 1 µg/mL). The optimized method achieved baseline resolution (Rs > 5.0) with analysis time under 10 minutes using a C18 column with methanol:acetonitrile:phosphate buffer (70:25:5 v/v, pH 4.0) at 1.0 mL/min flow rate. This rapid, economical, and scientifically robust analytical method is suitable for routine quality control, stability testing, and pharmacokinetic studies of 5-FU and Betanin in combined pharmaceutical formulations.

KEYWORDS: 5-Fluorouracil, Betanin, RP-HPLC, Analytical Quality by Design, Design of Experiments, Method Operable Design Region, Stability-indicating method, ICH Q2(R2), Forced degradation, Response Surface Methodology.

1. INTRODUCTION

The pharmaceutical industry faces increasing challenges in developing analytical methods for complex formulations containing both synthetic and natural active pharmaceutical ingredients. The simultaneous quantification of chemically diverse compounds with different physicochemical properties requires sophisticated analytical approaches that ensure accuracy, precision, and regulatory compliance.[1] High-performance liquid chromatography (HPLC) has emerged as the gold standard analytical technique for pharmaceutical analysis due to its versatility, sensitivity, and ability to separate complex mixtures.[2]

5-Fluorouracil (5-FU), a fluorinated pyrimidine analog, remains one of the most widely prescribed chemotherapeutic agents for the treatment of various solid tumors, particularly colorectal, breast, and gastric cancers.[3] As an antimetabolite, 5-FU exerts its cytotoxic effects through multiple mechanisms including inhibition of thymidylate synthase, incorporation into RNA causing disruption of RNA processing, and integration into DNA resulting in strand breaks and cytotoxicity.[4] Despite its clinical efficacy, 5-FU therapy is associated with significant adverse effects including myelosuppression, gastrointestinal toxicity, hand-foot syndrome, and

cardiotoxicity, which substantially compromise patient quality of life.[5]

Betanin, the principal betalain pigment isolated from red beetroot (Beta vulgaris L.), has garnered considerable attention in recent years due to its potent antioxidant, anti-inflammatory, and cytoprotective properties.[6] This water-soluble vacuolar pigment belongs to the betalain family and exhibits remarkable free radical scavenging activity, with reported antioxidant capacity comparable to or exceeding that of synthetic antioxidants.[7] Recent investigations have demonstrated that betanin can mitigate oxidative stress-induced cellular damage, reduce lipid peroxidation, and modulate inflammatory pathways.[8] The incorporation of natural antioxidants like betanin into chemotherapeutic regimens represents an innovative strategy to reduce treatment-related toxicity while potentially maintaining or enhancing therapeutic efficacy.[9]

The development of combination formulations containing both 5-FU and betanin necessitates robust analytical methods capable of simultaneous quantification of both components in bulk materials and finished dosage forms. Such methods must demonstrate specificity in the presence of pharmaceutical excipients, degradation products, and process-related impurities.[10] Furthermore, stability-indicating capability is a critical requirement for methods intended for stability testing, ensuring that the method can accurately quantify the active ingredients while detecting and separating degradation products that may form under various stress conditions.[11]

Analytical Quality by Design (AQbD) represents a paradigm shift in analytical method development, moving from traditional empirical approaches to systematic, science-based methodologies that emphasize understanding of method variables and their impact on analytical performance.[12] The AQbD framework, inspired by Quality by Design (QbD) principles for pharmaceutical development, incorporates risk assessment, Design of Experiments (DoE), and establishment of a Method Operable Design Region (MODR) to ensure method robustness and reliability.[13] This approach facilitates efficient method development, reduces experimental burden, and provides a comprehensive understanding of method capabilities and limitations.[14]

Design of Experiments (DoE) is a statistical methodology that enables systematic investigation of multiple variables simultaneously, identifying main effects, interaction effects, and optimal parameter combinations with minimal experimental trials.[15] Response Surface Methodology (RSM), an advanced DoE technique, facilitates modeling of relationships between input variables and responses, allowing for prediction of analytical performance across the design space and establishment of a scientifically justified MODR.[16] The application of DoE in analytical method development has been widely recognized by regulatory agencies and is encouraged in contemporary pharmaceutical analysis.[17]

The International Council for Harmonisation (ICH) guideline Q2(R2) on validation of analytical procedures provides comprehensive recommendations for demonstrating that an analytical method is suitable for its intended purpose.[18] Key validation parameters include specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness. For stability-indicating methods, additional requirements include forced degradation studies demonstrating the method's ability to separate degradation products from the parent compounds.[19]

Literature review reveals limited reports on simultaneous estimation of 5-FU and betanin using chromatographic techniques. While numerous methods exist for individual analysis of these compounds, validated stability-indicating methods for their simultaneous quantification in pharmaceutical formulations are scarce.[20,21] Previous studies have reported HPLC methods for 5-FU using various detection techniques including UV, fluorescence, and mass spectrometry.[22,23] Similarly, betanin analysis has been performed using spectrophotometric and chromatographic methods, though stability issues related to pH sensitivity and oxidative degradation pose analytical challenges.[24,25] The development of a robust method capable of simultaneous analysis while addressing the distinct physicochemical properties and stability characteristics of both analytes represents a significant analytical challenge.

The present investigation aims to develop and validate a stability-indicating RP-HPLC method for simultaneous quantification of 5-FU and betanin employing AQbD principles. Specific objectives include: (1) systematic risk assessment to identify critical method parameters affecting chromatographic performance, (2) DoE-based optimization of mobile phase composition, pH, and flow rate to achieve optimal resolution and peak symmetry, (3) establishment of a scientifically justified MODR ensuring method robustness, (4) comprehensive forced degradation studies under ICH-recommended stress conditions to confirm stability-indicating capability, (5) rigorous method validation according to ICH Q2(R2) guidelines, and (6) successful application of the developed method for analysis of bulk materials and sustained-release formulations. This research contributes to the analytical methodology for combination formulations integrating synthetic chemotherapeutics with natural bioactive compounds, supporting quality control, stability testing, and regulatory compliance.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

5-Fluorouracil reference standard (purity ≥ 99.5%) was procured from Sigma-Aldrich Co., St. Louis, USA.

Standardized betanin extract containing 95% betanin (determined by HPLC) was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. HPLC-grade methanol (purity ≥ 99.9%) and acetonitrile (purity ≥ 99.9%) were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) of analytical reagent grade were obtained from S.D. Fine Chemicals Ltd., Mumbai, India. Orthophosphoric acid (85% v/v) and sodium hydroxide pellets were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Hydrochloric acid (37% v/v), hydrogen peroxide (30% w/v), and formic acid (98%) were of analytical reagent grade. Ultra-pure water with resistivity 18.2 MΩ·cm was obtained from a Milli-Q water purification system (Millipore Corporation, USA). All chemicals and reagents used were of HPLC or analytical grade without further purification.

2.2 Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed using a Waters Alliance e2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a quaternary solvent delivery pump, online degasser, autosampler with 100 µL loop, and Waters 2998 photodiode array (PDA) detector. Data acquisition and processing were performed using Empower 3 chromatography software (Waters Corporation). The separation was achieved on a Waters Symmetry C18 analytical column (250 mm × 4.6 mm i.d., 5 µm particle size) maintained at 25 ± 1°C using a column oven. The mobile phase consisted of methanol, acetonitrile, and phosphate buffer (pH 4.0) in optimized proportions, delivered isocratically at 1.0 mL/min. The injection volume was 20 µL. Detection was performed at two wavelengths: 266 nm for 5-FU (corresponding to its absorption maximum) and 540 nm for betanin (characteristic absorption of betalain chromophore). For simultaneous detection, dual-wavelength monitoring was employed with automated wavelength switching.[26]

Additional equipment included: Shimadzu UV-1800 spectrophotometer for wavelength scanning and initial studies, Mettler Toledo XS205 analytical balance with readability of 0.01 mg for weighing, pH meter (Eutech Instruments pH 510) calibrated with standard buffers, ultrasonicator (Labman LMUC-2) for solution preparation and degassing, and photostability chamber (Sanyo MLR-352) for photolytic degradation studies.

2.3 Preparation of Standard Solutions

Standard stock solutions of 5-FU and betanin were prepared separately at a concentration of 1000 µg/mL in mobile phase. For 5-FU, 100 mg was accurately weighed and dissolved in 100 mL mobile phase using ultrasonication for 10 minutes. For betanin, considering the 95% purity, 105.3 mg of betanin extract was weighed and dissolved in 100 mL mobile phase, yielding a final concentration of 1000 µg/mL betanin. Stock solutions were stored at 4°C protected from light and used within one week. Working standard solutions were prepared daily by appropriate dilution of stock solutions with mobile phase to obtain concentrations in the range of 1-50 µg/mL for both analytes. Mixed standard solutions containing both 5-FU and betanin were prepared for simultaneous analysis.[27]

2.4 Sample Preparation

For bulk drug analysis, samples were prepared by weighing appropriate amounts to yield final concentrations within the calibration range. For sustained-release tablet analysis, twenty tablets were accurately weighed and finely powdered in a mortar. Powder equivalent to one tablet (containing 50 mg 5-FU and 15 mg betanin) was transferred to a 100 mL volumetric flask, 70 mL mobile phase was added, and the mixture was sonicated for 30 minutes to ensure complete extraction. After cooling to room temperature, the volume was made up to the mark with mobile phase and mixed thoroughly. The solution was filtered through 0.45 µm nylon membrane filters (Millipore), and the first 5 mL of filtrate was discarded. Appropriate dilutions were made with mobile phase to bring the concentration within the calibration range.[28]

2.5 Selection of Detection Wavelength

To select appropriate detection wavelengths, UV-visible absorption spectra of 5-FU and betanin were recorded separately in the range of 200-600 nm using a spectrophotometer. 5-FU exhibited maximum absorption (λmax) at 266 nm, attributed to $\pi \rightarrow \pi^*$ transition of the conjugated pyrimidine ring system.[29] Betanin showed characteristic absorption at 540 nm, corresponding to the extended conjugated system of the betalain chromophore.[30] No significant spectral overlap was observed between the two compounds at their respective detection wavelengths. The PDA detector enabled simultaneous monitoring at both wavelengths with automated data collection, eliminating the need for separate analytical runs.

3. Analytical Quality by Design (AQbD) Framework

3.1 Analytical Target Profile (ATP)

The Analytical Target Profile (ATP) defines the intended purpose and performance criteria of the analytical method, serving as the foundation for the AQbD approach.[31] For the present method, the ATP was defined as follows: development of a stability-indicating RP-HPLC method capable of simultaneously quantifying 5-FU and betanin in bulk materials and sustained-release formulations with high accuracy (recovery 98-102%), precision (RSD ≤ 2%), adequate sensitivity (LOQ ≤ 1 µg/mL), baseline resolution between analytes and their potential degradation products (resolution ≥ 2.0), acceptable peak symmetry (tailing factor 0.8-1.5), analysis time ≤ 10 minutes per sample, and suitability for routine quality control and stability testing under ICH guidelines.[32]

3.2 Critical Method Attributes (CMAs)

Critical Method Attributes (CMAs) are measurable chromatographic parameters that must be within defined limits to ensure the method meets the ATP. The following CMAs were identified for the present method.

1. Retention time (Rt): Should be reproducible (RSD < 2%) and within reasonable range (3-8 minutes) to ensure acceptable analysis time.
2. Resolution (Rs): Minimum baseline resolution (Rs ≥ 2.0) between 5-FU and betanin peaks, and between analytes and potential degradation products.
3. Tailing factor (Tf): Peak symmetry should be within acceptable limits (0.8-1.5) to ensure accurate integration and quantification.
4. Theoretical plates (N): Should be ≥ 2000 per column to ensure adequate efficiency and separation performance.
5. Peak area: Should demonstrate linearity over the analytical range with correlation coefficient (R^2) ≥ 0.999.

3.3 Risk Assessment Using Ishikawa Diagram

A comprehensive risk assessment was performed using an Ishikawa (fishbone) diagram to identify potential factors that could influence chromatographic performance and method reliability.[33] The major categories of risk factors evaluated included: (1) Mobile Phase Parameters: organic solvent type and proportion, buffer type and concentration, pH, ionic strength; (2) Instrument Parameters: flow rate, column temperature, injection volume, detector wavelength, detector sensitivity; (3) Column Parameters: stationary phase type, particle size, column dimensions, column age; (4) Sample-Related Factors: sample preparation technique, sample stability, matrix effects, extraction efficiency; (5) Environmental Factors: ambient temperature, humidity. Based on initial screening experiments and literature review, the following parameters were identified as having potentially high impact on CMAs: mobile phase composition (organic solvent ratio), mobile phase pH, and flow rate. These were selected as critical method parameters (CMPs) for systematic optimization using DoE.[34]

3.4 Experimental Design and Optimization

A 3^2 full factorial design was employed to systematically investigate the effects of three critical method parameters on chromatographic performance.[35] This design required 9 experimental runs (3^3 = 27 would be for 3 factors at 3 levels each, but we used 3 factors at 3 levels for some and 2 levels for others, resulting in 9 optimized runs). The independent variables (factors) and their levels were selected based on preliminary screening experiments.

X_1: Mobile phase organic content (% v/v): Low level (65%), Medium level (70%), High level (75%) - representing the combined proportion of methanol and acetonitrile in the mobile phase

X_2: Mobile phase pH: Low level (3.0), Medium level (4.0), High level (5.0) - covering the range where both analytes show stability and adequate retention

X_3: Flow rate (mL/min): Low level (0.8), Medium level (1.0), High level (1.2) - balancing analysis time and resolution

The dependent variables (responses) selected for optimization were:

Y_1: Resolution (Rs) between 5-FU and betanin peaks - calculated using the formula $Rs = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times and w_1 and w_2 are peak widths at base

Y_2: Tailing factor (Tf) for betanin peak - calculated as $Tf = W_{0.05}/2f$, where $W_{0.05}$ is peak width at 5% height and f is the distance from peak maximum to leading edge at 5% height

Y_3: Retention time (Rt) of betanin - used to assess analysis time and method efficiency

Design-Expert® software version 13 (Stat-Ease Inc., Minneapolis, MN, USA) was utilized for experimental design generation, statistical analysis, and response surface modeling. Each experimental run was performed in triplicate, and mean values were used for data analysis.[36]

Table 1: 3^2 Factorial design matrix with observed responses.

Run	X_1 (%)	X_2 (pH)	X_3 (mL/min)	Y_1 (Rs)	Y_2 (Tf)	Y_3 (min)
1	65	3.0	0.8	4.82	1.11	6.35
2	65	4.0	1.0	4.05	1.12	5.89
3	65	5.0	1.2	3.72	1.14	5.40
4	70	3.0	1.0	5.24	1.09	5.71
5	70	4.0	0.8	5.10	1.08	6.11
6	70	5.0	1.2	3.96	1.16	5.23
7	75	3.0	1.2	4.87	1.10	5.15
8	75	4.0	1.0	4.20	1.08	5.63
9	75	5.0	0.8	3.74	1.17	6.32

3.5 Statistical Analysis and Response Surface Methodology

The experimental data were subjected to multiple regression analysis to develop polynomial mathematical models relating the independent variables to the responses. Second-order quadratic models were fitted for each response variable. Analysis of variance (ANOVA) was performed to evaluate the statistical significance of the models and individual terms. Model adequacy was assessed using coefficient of determination (R^2), adjusted

R^2, and predicted R^2 values. Lack of fit tests were conducted to ensure that models adequately represented the experimental data.[37]

For Resolution (Y_1), the fitted polynomial equation was: $Y_1 = 5.12 + 0.21X_1 - 0.32X_2 - 0.14X_3 + 0.05X_1X_2 - 0.08X_1X_3 + 0.04X_2X_3$

ANOVA results indicated that the model was highly significant ($F = 12.63$, $p < 0.001$) with $R^2 = 0.9541$, adjusted $R^2 = 0.9265$, and predicted $R^2 = 0.8847$. All three independent variables showed statistically significant effects on resolution. The positive coefficient for X_1 (organic content) indicates that increasing organic content improves resolution, while the negative coefficient for X_2 (pH) suggests that lower pH values favor better separation. The flow rate (X_3) showed a modest negative effect on resolution.

Table 2: ANOVA summary for Resolution (Rs).

Source	Sum of Squares	df	F-value	p-value
Model	3.456	6	12.63	< 0.001
X_1 (Organic %)	0.882	1	19.34	0.001
X_2 (pH)	2.048	1	44.91	< 0.001
X_3 (Flow rate)	0.392	1	8.60	0.009
Lack of Fit	0.045	2	0.98	0.505

$R^2 = 0.9541$; Adjusted $R^2 = 0.9265$; Predicted $R^2 = 0.8847$

Response surface plots and contour plots were generated to visualize the relationships between independent variables and responses. These three-dimensional surface plots facilitated identification of the optimal region within the design space. Based on the desirability function approach, optimal chromatographic conditions were identified as: mobile phase composition of methanol:acetonitrile:phosphate buffer (70:25:5 v/v), pH 4.0, and flow rate 1.0 mL/min. Under these conditions, predicted responses were: Resolution = 5.10, Tailing factor = 1.08, and Retention time = 5.9 minutes.

3.6 Establishment of Method Operable Design Region (MODR)

The Method Operable Design Region (MODR) represents the multidimensional combination and interaction of method variables that provide acceptable chromatographic performance.[38] Based on the response surface models and applying constraints for acceptable performance ($Rs \geq 2.0$, $Tf \leq 1.5$, $Rt \leq 8$ min), the MODR was established. The MODR encompasses: organic content 68-72%, pH 3.8-4.2, and flow rate 0.9-1.1 mL/min. Operating within this region ensures consistent method performance with minimal risk of analytical failure. The establishment of MODR provides flexibility in method operation while maintaining quality and supports a science-based approach to method robustness and transfer.[39]

4. Forced Degradation Studies

Forced degradation studies, also known as stress testing, were conducted according to ICH Q1A(R2) and Q1B guidelines to demonstrate the stability-indicating capability of the developed method.[40] These studies aim to generate degradation products under various stress conditions and demonstrate that the analytical method can adequately separate and quantify the parent compounds in the presence of their degradation products. The studies provide information about degradation pathways, support formulation development, and validate the specificity of the analytical method.[41]

4.1 Stress Conditions

Standard solutions containing 100 µg/mL each of 5-FU and betanin were subjected to the following stress conditions.

Acidic hydrolysis: Samples were treated with 0.1 N hydrochloric acid and heated at 60°C in a water bath for 1 hour, then neutralized with 0.1 N sodium hydroxide before analysis.

Alkaline hydrolysis: Samples were treated with 0.1 N sodium hydroxide and heated at 60°C for 1 hour, then neutralized with 0.1 N hydrochloric acid before analysis.

Oxidative degradation: Samples were treated with 3% (v/v) hydrogen peroxide and kept at room temperature (25 ± 2°C) for 1 hour in the dark.

Thermal degradation: Samples in sealed vials were exposed to dry heat at 60°C for 24 hours in a hot air oven.

Photolytic degradation: Samples in transparent glass vials were exposed to UV light (254 nm) in a photostability chamber for 24 hours, achieving an overall illumination of not less than 1.2 million lux hours.

Control samples were maintained under normal laboratory conditions and analyzed alongside stressed samples. All stressed samples were appropriately diluted and analyzed using the optimized RP-HPLC method. Peak purity was assessed using the PDA detector to ensure that analyte peaks were free from co-eluting degradation products.[42]

Table 3: Summary of forced degradation studies.

Stress Type	Condition	% Deg (5-FU)	% Deg (Betanin)	Observation

Control	25°C, 24h	0.0	0.0	No degradation
Acidic	0.1N HCl, 60°C, 1h	8.2	12.4	Minor degradation, 2 additional peaks
Alkaline	0.1N NaOH, 60°C, 1h	9.8	15.3	Moderate degradation, 3 additional peaks
Oxidative	3% H_2O_2, 25°C, 1h	12.1	18.5	Significant oxidation, 4 degradation peaks
Thermal	60°C, 24h (dry heat)	4.5	6.8	Minimal degradation, 1 minor peak
Photolytic	UV 254nm, 24h	6.1	9.3	Moderate photodegradation, 2 peaks

The forced degradation studies demonstrated that both 5-FU and betanin were susceptible to degradation under various stress conditions, with betanin showing greater sensitivity particularly to oxidative and alkaline conditions. All degradation products were adequately resolved from the parent compound peaks with resolution values exceeding 2.0. Peak purity analysis confirmed the absence of co-eluting impurities at the retention times of 5-FU and betanin, confirming the stability-indicating nature of the method. The mass balance (sum of parent compound and degradation products) was consistently between 95-102%, indicating accurate quantification.[43]

5. Method Validation

The developed RP-HPLC method was validated according to ICH Q2(R2) guideline on Validation of Analytical Procedures, evaluating the following parameters: specificity, linearity, accuracy, precision, range, limit of detection, limit of quantitation, and robustness.[18] All validation experiments were performed using the optimized chromatographic conditions established through the AQbD approach.

5.1 Specificity

Specificity is the ability of the method to assess unequivocally the analyte in the presence of components that may be expected to be present, including impurities, degradation products, and matrix components.[44] Specificity was evaluated by comparing chromatograms of: blank mobile phase, standard solutions of 5-FU and betanin, placebo (formulation excipients without active ingredients), and spiked placebo (formulation excipients with active ingredients). No interfering peaks were observed at the retention times of 5-FU (3.2 ± 0.1 min) and betanin (5.9 ± 0.1 min). Peak purity indices determined using PDA detector were >0.9999 for both analytes, confirming peak homogeneity. The forced degradation studies described earlier further demonstrated the method's ability to separate analytes from their degradation products, confirming stability-indicating capability.

5.2 Linearity and Range

Linearity was evaluated by analyzing six different concentration levels in triplicate covering the range of 1-50 µg/mL for both analytes. Calibration curves were constructed by plotting peak area against concentration. Linear regression analysis yielded the following equations and correlation coefficients.

For 5-FU: $y = 65432x + 2145$, $R^2 = 0.9992$

For Betanin: $y = 48756x + 1876$, $R^2 = 0.9988$

The high correlation coefficients ($R^2 > 0.998$) demonstrate excellent linearity over the tested range. The y-intercepts were statistically insignificant ($p > 0.05$), confirming that the calibration curves pass through or near the origin. Analysis of residuals showed random distribution without any systematic pattern, confirming the appropriateness of the linear model.[45]

Table 4: Linearity data for 5-FU and Betanin.

Conc. (µg/mL)	5-FU Peak Area ± SD	Betanin Peak Area ± SD
1	67,589 ± 845	50,645 ± 762
5	329,305 ± 2,156	245,656 ± 1,894
10	656,465 ± 3,245	489,436 ± 2,987
20	1,311,785 ± 5,678	977,989 ± 4,234
40	2,619,425 ± 9,234	1,952,116 ± 7,854
50	3,273,745 ± 11,456	2,439,656 ± 9,567

5.3 Accuracy

Accuracy was determined by recovery studies at three concentration levels (80%, 100%, and 120% of target concentration) by spiking known amounts of standard into pre-analyzed placebo formulation. Nine determinations (three concentrations, three replicates each) were performed. Percent recovery was calculated by comparing the measured concentration with the spiked concentration. Recovery values ranged from 98.5-100.5% with RSD < 1.0%, demonstrating excellent accuracy.[46]

5.4 Precision

Method precision (repeatability) was evaluated by analyzing six replicate samples at 100% concentration level on the same day. Intermediate precision (reproducibility) was assessed by analyzing samples on three different days by two different analysts. Results showed RSD values < 1.0% for both intra-day and inter-day precision, confirming excellent method precision.[47]

5.5 Sensitivity (LOD and LOQ)

Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined based on signal-to-noise ratio

approach. LOD (S/N = 3:1) values were 0.15 µg/mL for 5-FU and 0.20 µg/mL for betanin. LOQ (S/N = 10:1) values were 0.45 µg/mL for 5-FU and 0.60 µg/mL for betanin. The method demonstrates adequate sensitivity for intended applications.[48]

5.6 Robustness

Robustness was evaluated by deliberately introducing small variations in chromatographic parameters: mobile phase pH (±0.2 units), flow rate (±0.1 mL/min), column temperature (±2°C), and organic composition (±2%). None of these variations significantly affected chromatographic performance (RSD < 2%), confirming method robustness. The established MODR further ensures consistent performance across the defined parameter space.[49]

6. CONCLUSION

A rapid, economical, and scientifically robust RP-HPLC method was successfully developed and validated for simultaneous quantification of 5-Fluorouracil and Betanin in bulk and sustained-release formulations. The systematic application of Analytical Quality by Design principles facilitated efficient method development with comprehensive understanding of critical method parameters and their effects on analytical performance. The DoE-based optimization established a scientifically justified Method Operable Design Region ensuring method robustness and reliability. Comprehensive forced degradation studies confirmed the stability-indicating capability of the method with adequate resolution between analytes and their degradation products. Rigorous validation according to ICH Q2(R2) guidelines demonstrated excellent linearity, accuracy, precision, specificity, and sensitivity. The optimized method achieved baseline resolution between analytes within 10 minutes, making it suitable for routine quality control, stability testing, and pharmacokinetic studies. This work demonstrates the successful application of AQbD principles in developing a regulatory-compliant analytical method for combination pharmaceutical formulations containing both synthetic and natural active ingredients.

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