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Development and Validation of a QbD-Based UV Spectrophotometric Method for the Quantification of Silibinin Using Area Under the Curve (AUC) Approach

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KEYWORDS

Silibinin, UV Spectrophotometer, Area under curve, Method validation, QbD, ICH guidelines

ABSTRACT:

Introduction: Reliable quantification of silibinin, a therapeutically significant flavonolignan, is essential for pharmaceutical analysis. A UV spectrophotometric method based on the Area Under the Curve (AUC) principle offers a simple, economical, and robust approach by minimizing wavelength-dependent variability. Integration of a Quality by Design (QbD) framework enables systematic method optimization and ensures reproducible and regulatory-compliant analysis.

Objectives: The aim of this study was to develop and validate a simple, rapid, and reliable UV spectrophotometric method based on the Area Under the Curve principle for the quantitative estimation of silibinin. The method was designed using a Quality by Design approach to ensure accuracy, precision, and robustness suitable for routine pharmaceutical analysis.

Methods: Absorbance readings were recorded within the wavelength range of 265.60 to 311.80 nanometres, with 289 nanometres selected as the central wavelength for AUC calculations. A Central Composite Design was applied to identify and optimise critical method parameters and to understand their influence on analytical performance. Following optimization, the method was validated according to the International Council for Harmonisation Q2 R1 guidelines, including assessments of linearity, accuracy, precision, and robustness

Results: The Quality by Design framework supported a systematic evaluation of analytical variables and facilitated the development of a stable and efficient method. The Central Composite Design enabled efficient screening of factors and optimisation of conditions. The method exhibited excellent linearity across the concentration range of 2 to 16 micrograms per millilitre, with a correlation coefficient of 0.9997. Precision studies demonstrated strong repeatability and reproducibility, with the coefficient of variation remaining below two percent. The method also showed good sensitivity and specificity, ensuring accurate quantification without interference. Validation results confirmed compliance with International Council for Harmonisation criteria.

Conclusions: The developed UV AUC method is simple, cost effective, and analytically sound. The Quality by Design based optimisation ensures robustness and consistency, making the method suitable for routine quality control and quantitative analysis of silibinin in pharmaceutical formulations.

1. Introduction

Silibinin is a naturally occurring flavonolignan a naturally occurring substance that was taken from the milk thistle plant's (Silybum marianum) seeds [1]. It is the primary active component of silymarin and has garnered significant attention due to its potent hepatoprotective properties. The mechanisms underlying its therapeutic effects are multifaceted,

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involving inhibition of lipid peroxidation, enhancement of antioxidant defenses, and membrane stabilization [2, 3].

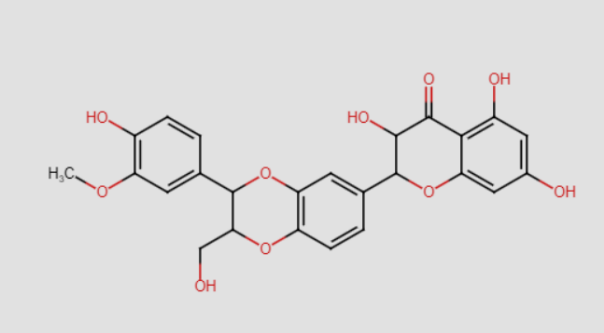

Figure 1. Structure of Silibinin (3, 5, 7-trihydroxy-2-[3-(4-hydroxy-3-methoxy phenyl)-2-(hydroxy-methyl)-1, 4-bezodioxan-6-yl]-4-chromanone).

Silibinin can be analyzed utilizing a range of analytical techniques, including TLC and HPLC, and UV-visible spectrophotometry [4]. Among these, UV spectrophotometric methods are widely preferred in quality control settings due to their simplicity, cost-effectiveness, and rapid analysis. In the current study, a novel and robust UV spectrophotometric method employing the Area Under Curve was developed and validated for the quantification of silibinin. The method utilizes absorbance measurements in the range of 265.60 nm to 311.80 nm, with 289 nm serving as the central wavelength. This technique proved to be accurate, sensitive, specific, and reproducible, with a coefficient of variation (CV) of less than 2%, making it highly suitable for routine analysis [5-7].

To ensure method robustness and performance reliability, the Quality by Design (QbD) approach was employed throughout method development. QbD emphasizes the importance of predefined objectives and thorough understanding of process variables, leading to the establishment of a design space that ensures method consistency and quality [8], In Within this framework, a 2^3 optimal design, categorized under response surface methodology (RSM) was applied to evaluate the influence of multiple factors on analytical performance. Key variables such as wavelength range, solvent composition, and sample concentration were systematically varied to assess their individual and interaction effects on absorbance response and method robustness [9].

The use of 2^3 optimal design enabled the identification of optimal conditions for accurate silibinin quantification while minimizing experimental runs, thus saving time and resources. Statistical analysis 2^3 optimal design results demonstrated significant model predictability and a high degree of linearity (R^2 = 0.9997) concentration range of 2–16 µg/mL. Furthermore, the method was validated as per International Council for Harmonisation (ICH) guidelines, addressing critical parameters including accuracy, precision, linearity, specificity, and reproducibility [10].

Overall, the developed AUC-based UV spectrophotometric method, guided by QbD principles and optimized via 2^3 optimal design, presents a highly efficient and reliable alternative to conventional methods for silibinin analysis. It offers a flexible, rapid, and scientifically sound analytical solution that can be easily adapted for quality control applications in pharmaceutical and herbal formulation industries [11, 12].

2. Objectives

The objective of the present investigation was to develop and validate a simple, rapid, and dependable UV spectrophotometric method based on the Area Under the Curve (AUC) approach for the quantitative determination of silibinin in pharmaceutical formulations. The method was systematically developed using a Quality by Design (QbD) framework to achieve a comprehensive understanding of critical method variables, ensure consistent analytical performance, and enhance robustness for routine quality control use.

Absorbance data were collected over the wavelength range of 265.60–311.80 nm, with 289 nm selected as the central wavelength for AUC measurement based on the characteristic UV absorption profile of silibinin. A 2^3 optimal design was applied to identify critical method parameters and to evaluate both their individual and interactive effects on the analytical responses. This multivariate optimization strategy facilitated the establishment of a well-defined design space, thereby reducing analytical variability and improving overall method reliability.

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Following optimization, the developed method was validated in accordance with International Council for Harmonisation (ICH) Q2 (R1) guidelines. Validation studies demonstrated satisfactory linearity, accuracy, precision, and robustness, confirming that the proposed method is suitable for the accurate and reproducible estimation of silibinin in routine pharmaceutical analysis.

3. Methods

All spectral measurements were performed using a Shimadzu UV Spectrophotometer 1700 (UVProbe 2.21) equipped with a 1.0 cm matched quartz cell. Silibinin (purity ≥98.0%) was procured from Merck Co., Ltd. (Belgium). HPLC-grade methanol (≥99.9%) Thermo Fisher Scientific's Milli-Q water was utilized continuously throughout the experiment, and Merck Ltd. (Mumbai, India) supplied the formic acid (purity ≥98.0%). No additional purification was necessary because all analytical-grade chemicals and reagents were used precisely as supplied.

Preparation of standard stock solution (100 µg/ mL)

An accurately weighed 10 mg of Silibinin was transferred to a 100 mL volumetric flask, dissolved in DMSO, and manually shaken for 10 minutes. The solution was then diluted to volume with distilled water to obtain a final concentration of 100 µg/mL.

DoE- Method Optimization and Response Surface Analysis

Optimization of method was systematically conducted with Stat-Ease Inc.'s Design-Expert® software (Version 13.0, USA) employing a 2^3, a response surface methodology (RSM) approach. Prior to multivariate analysis, critical method parameters and their suitable operational ranges identified through preliminary univariate assessments and evaluations. Based on these findings, two independent variables wavelength and scanning interval were selected for further optimization. Each factor was evaluated at three levels (−1, 0, +1), resulting in a total of 11 experimental runs according to the 2^3 optimal design shown in Table 1. The performance of each run was evaluated using key response variables: scanning interval and wavelength.Statistical analysis of the model was performed using analysis of variance (ANOVA) assess the adequacy and significance of regression model. In addition, MLRA was applied to assess both the primary and interaction effects of the chosen factors on the measured responses. This structured, multivariate optimization strategy enabled the identification of absorbance, ensuring improved characteristics across experiments [13].

Table 1. Experimental matrix for optimal design.

	Factor 1	Factor 2	Response 1
Run	A:Wavelength	B:Scanning interval	Absorbance
1	291	2	0.382
2	287	1	0.362
3	289	2	0.377
4	291	1	0.38
5	287	1	0.361
6	289	0.2	0.367
7	291	0.2	0.379
8	289	0.2	0.371
9	287	0.2	0.351
10	289	1	0.375
11	287	2	0.375

Quality-by-Design (QbD)-Enable UV Method Development and Optimization

The RSM design matrix was created using Design-Expert® software (Version 13.0), which supports an organized approach to method optimization. Using ANOVA, model appropriateness and predictive validity were evaluated, emphasizing the identification of statistically significant models with high predictive accuracy. Key statistical metrics including model F-values, p-values, regression coefficients, and lack-of-fit tests were evaluated to ensure model robustness and reliability. The comprehensive statistical results are given in Table 2, providing important information about the model's functionality, importance, and capacity for prediction. These findings confirm the adequacy of the selected quadratic models in accurately describing the relationships between the independent variables and the observed responses [14].

Design-Expert® software (Version 13.0) was employed to generate the experimental matrix for (RSM), enabling a methodical and organized approach to

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optimization process. The overall suitability of the model were evaluated using analysis of variance , with particular focus on identifying statistically significant models, thus confirming their robustness in accurately predicting observed responses. Tables 2 contain comprehensive statistical results, such as regression coefficients, lack-of-fit analysis, and model significance. These tables offer crucial information about the model's dependability, performance, and predictive abilities.

Second-order polynomial equations developed to quantitatively explain the connection between the response and the independent factors: absorbance (X_1), scanning interval (X_2),.The sign of each regression coefficient indicates the nature of the variable's effect: positive coefficients represent synergistic (enhancing) impact , whereas negative coefficients indicate antagonistic (inhibitory) effects. The absolute magnitude of each coefficient indicates the relative impact of the corresponding variable on the response corresponding factor on the response. The derived models exhibited excellent conformity with experimental findings, highlighting their prognostic validity and statistical robustness. These polynomial equations not only validated the optimization process but also provided mechanistic insights into the variable-response relationships. This quantitative framework enables informed adjustment of method parameters, facilitating the development of robust, UV methods and guiding future optimization strategies [15].

Polynomial equation:
RT=+0.3725+0.0089*A+0.0055*B-0.0053AB

Determination of λmax and selection of wavelengths

Standard Drug Solution (standard stock solution) In a volumetric flask of 10 mL, 10 mg of precisely weighed drug was added. The drug was dissolved in 10mL of DMSO, to achieve a concentration of 100ug/mL. A UV spectral scan was performed on the solution 800- 200 nm. SLY showed maximum absorbance at 289 nm. (Figure 4)

Validation

Validation of the developed method was performed in compliance with ICH guidelines and different parameters were evaluated for the same such as Linearity, Correlation Coefficient, LOD, LOQ, Precision, Accuracy, Robustness and Ruggedness [16-23].The data pertaining to the above parameters is reported below in table.

Linearity

Aliquots ranging from 0.5 to 2.5 mL were withdrawn from the stock standard solution and transferred into five separate 10 mL volumetric flasks. Each flask was diluted to volume with the same solvent to obtain final concentrations ranging from 2 to 16 µg/mL. The Area Under the Curve (AUC) for each concentration was recorded within the selected wavelength range.

Accuracy and precision

Accuracy of proposed method was assessed through recovery studies performed at three concentration levels: 50%, 100%, and 150%. At each level, a known amount of standard drug was spiked into the pre-analyzed sample solution, and the Area Under the Curve (AUC) was measured at the predetermined wavelengths.

The precision of the method was evaluated in terms of repeatability, intra-day, and inter-day variations. Silibinin was analyzed six times under identical conditions at a concentration of 0.6 µg/mL in order to evaluate repeatability. (Table 3) By analyzing the 2-16 g/mL of silibinin three times in a single day, intra-day accuracy was ascertained. By analyzing the same concentration of the solutions every day for three days, the inter-day precision was ascertained. (Table 3)

Sensitivity

The sensitivity of the proposed method was determined by calculating the limit of detection (LOD) and limit of quantitation (LOQ). These parameters were estimated using the following equations,

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

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

Where, SD = Standard deviation and S = Slope of the corresponding calibration plot.

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Ruggedness

A homogenous sample's aliquots were analyzed to assess the suggested method's ruggedness while introducing deliberate minor variations, without altering the operational and environmental condition. (Table 3)

4. Results And Discussion

Optimized UV Method Conditions

The UV spectrophotometric method was refined through a systematic optimization process using the Central Composite Design in Design Expert software. The statistical evaluation showed that all response surface models were reliable and meaningful, with strong regression values and appropriate adequacy measures. The absence of significant lack of fit indicated that the models accurately represented the relationship between the variables and could be confidently used for prediction. This confirmed both the robustness and the suitability of the developed models for method optimization.

The optimization was carried out using the desirability function approach, which allowed multiple analytical parameters to be evaluated at the same time. The software generated several solutions with very high desirability values, many of which were close to or exactly equal to one. Such values indicate that the selected solutions achieved an excellent overall balance among the targeted responses. The design space overlay plot presented in Figure 3 clearly illustrates the allowable region where all analytical requirements were met. The yellow shaded portion of the plot marks the area in which both absorbance and scanning interval perform optimally, ensuring improved method efficiency and reliable measurements. Based on the desirability outcomes, the final optimized method conditions included an absorbance value of 0.37 along with a scanning interval that provided clear spectral resolution and reduced background noise. These settings were found to offer the best combination of sensitivity, precision, and consistency.To confirm the reliability of the optimized conditions, validation experiments were performed. The experimental results for all the selected responses differed by less than five percent from the values predicted by the model. This close agreement demonstrated the accuracy of the optimization and confirmed that the developed method performs consistently within the predicted design space, optimized UV spectrophotometric method is precise, reproducible, and well suited for routine use. The validated design space and high desirability value support its practical applicability and reinforce its suitability for analytical method development under Quality by Design principles.

Table 2. Overview of the analysis of variance (ANOVA) results for the experimental design concerning the proposed UV method.

	Sum of Squares	**df**	**Mean²**	**F-value**	**p-value**	
Model	0.0009	3	0.0003	52.96	< 0.001	S
A-Wavelength	0.0005	1	0.0005	96.60	< 0.001	
B-Scanning interval	0.0002	1	0.0002	36.97	< 0.001	
AB	0.0001	1	0.0001	19.85	0.003	
Residual	0.0000	7	5.612 E-06			
Lack of Fit	0.0000	5	6.157 E-06	1.45	0.456	N S
Pure Error	8.500E-06	2	4.250 E-06			
Cor Total	0.0009	10				

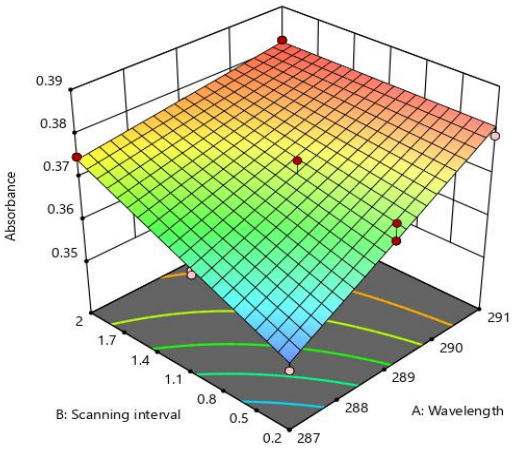

Figure 2. A three-dimensional response surface plot showing the impact on scanning interval (X2) and absorbance (X1).

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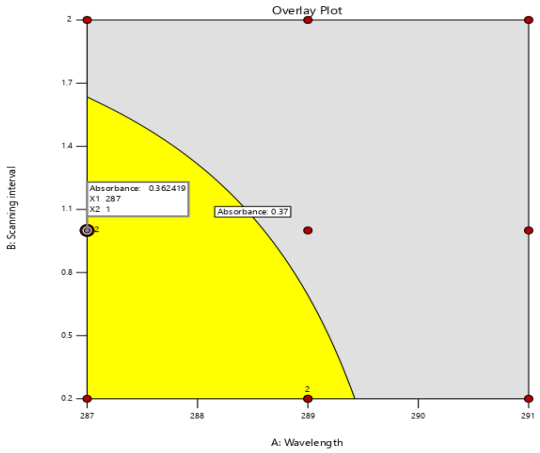

Figure 3. Design space overlay plot represents the optimized design space where all method criteria meet desired values.

Analytical Method Validation

The developed UV spectrophotometric methods were thoroughly validated in accordance with the International Council for Harmonisation Q2 R1 guidelines. All essential validation parameters were carefully examined to confirm that the methods are reliable, accurate, and suitable for the quantitative determination of silibinin in the tested samples.

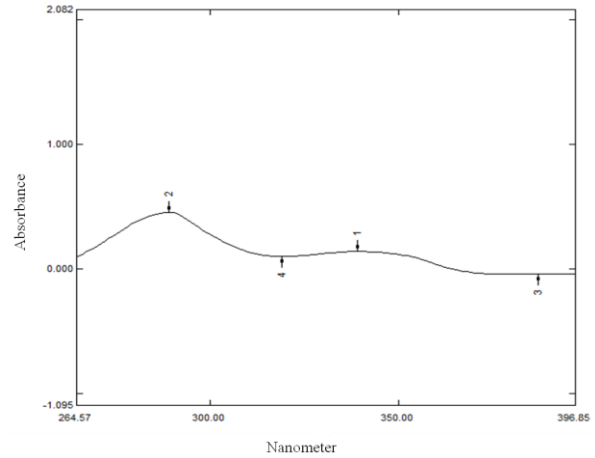

Figure 4. Spectrum of silibinin.

The validation process included an assessment of linearity, analytical range, limit of detection, limit of quantification, accuracy, precision for both intra day and inter day measurements, and robustness. These parameters were selected to provide a comprehensive understanding of the performance of the developed methods under routine analytical conditions.

Linearity was evaluated across a wide concentration range to ensure that the analytical response increased proportionally with the concentration of silibinin. The limit of detection and the limit of quantification were determined to establish the minimum amount of the analyte that could be reliably detected and quantified with acceptable accuracy and precision. Accuracy studies were performed by recovery experiments at multiple concentration levels to confirm that the methods consistently yielded results close to the true value.

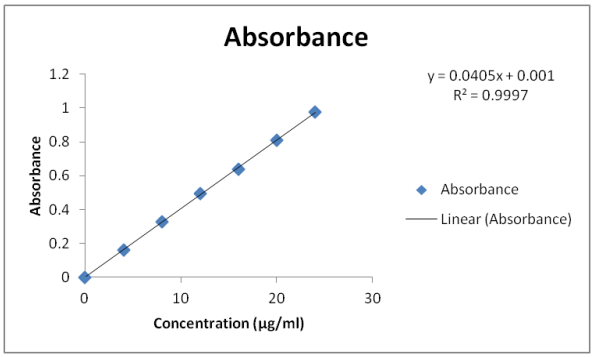

Figure 5. Standard calibration curve for Linearity of Silibinin by UV spectrophotometric method.

Precision was examined by repeated analysis within a single day and across different days. The low percent relative standard deviation values obtained for both intra day and inter day measurements demonstrated excellent repeatability and reproducibility. Robustness was evaluated by introducing small and deliberate variations in the experimental conditions, such as slight changes in wavelength, flow rate, or solvent composition. The minimal influence of these variations on the analytical response confirmed that the methods remain stable and dependable under routine laboratory conditions.

A detailed summary of all validation results is presented in Table 3. The data clearly confirm that UV spectrophotometric methods fulfil all acceptance criteria specified in the ICH guidelines. These findings demonstrate that the developed methods possess the required precision, accuracy, sensitivity, and robustness to support the reliable quantitative analysis of silibinin

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Linearity

The calibration curve prepared by plotting the peak area against the corresponding concentration of silibinin showed a clear and well defined linear relationship. The correlation coefficient R squared value of 0.9997 confirmed the excellent linearity of the method and demonstrated that the analytical response increased proportionally with the concentration of the analyte.A similar observation was made in the UV spectrophotometric study, where measurements obtained across the concentration range of 2 to 16 micrograms per millilitre also produced a correlation coefficient of 0.9997 as shown in Figure 5. This high level of linearity in both analytical techniques provides strong assurance that the method is reliable for the quantitative estimation of silibinin across the selected range. The results collectively indicate that the analytical response is consistent, predictable, and suitable for routine analysis.

Limit of Detection and Limit of Quantification

The sensitivity of the method was evaluated by calculating the limit of detection and the limit of quantification from the slope of the calibration curve and the standard deviation of the intercept. The limit of detection was found to be 12.046 micrograms per millilitre, while the limit of quantification was 3.975 micrograms per millilitre. These values indicate that the method is capable of detecting and accurately quantifying very small amounts of silibinin. The percent relative standard deviation for peak area measurements remained below two percent, which confirms that the method demonstrates satisfactory sensitivity and precision. The detailed values are provided in Table 3.

Precision: Intra day and Inter day Studies

Precision was assessed by evaluating both intra day and inter day variability across the concentration range of 2 to 16 micrograms per millilitre. Each concentration was analysed in triplicate to ensure reliability. The percent relative standard deviation values for both intra day and inter day measurements were consistently below two percent. These results indicate excellent repeatability within the same day and strong reproducibility across different days, confirming that the proposed method performs reliably under routine analytical conditions. The results are summarised in Table 3.

Table 3. Summary of validation parameters of Silibinin by UV

Sr. no	Validation parameters	Silibinin		
1	Linearity			
	Linearity range (µg/mL)	2-16		
	Correlation-coefficient	0.999		
2	LOD (µg/mL)	3.975		
	LOQ(µg/mL)	12.046		
3	System suitability			
	Absorbance (%RSD)	0.68		
4	Precision			
	Intra-day (%RSD)	Morning	Afternoon	Evening
		0.48	0.956	0.418
	Inter-day (%RSD)	DAY 1	DAY 2	DAY 3
		0.84	0.56	0.82
5	Robustness			
	Detection wavelength (%RSD)			
	286 nm	0.893%		
	288 nm	0.715%		
	290 nm	1.035%		
6	Accuracy			

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	50% recovery	99.02		
	100% recovery	100.83		
	150% recovery	99.33		
	Ruggedness			
	Analyst 1	1.359		
	Analyst 2	1.045		
	Change of Instrument	Shimadzu -1900	0.678	
		Shimadzu -1800	1.483	

Accuracy

Accuracy was examined through recovery studies at three concentration levels which represented 80, 100, and 150 percent of the target value. The percentage recovery ranged from 99.02 percent to 99.33 percent, demonstrating that the method consistently yielded results close to the true amount of analyte present. These findings comply with the International Council for Harmonisation Q2 R1 guidelines and affirm that the analytical method provides accurate and dependable results. The detailed outcomes are presented in Table 3.

Robustness

Robustness was evaluated by introducing small but deliberate variations in analytical conditions, specifically slight changes in wavelength. The method maintained stable performance under all altered conditions, with percent relative standard deviation values remaining below two percent. Recovery studies carried out at 50, 100, and 150 percent further supported the robustness of the UV method, demonstrating that the analytical response remained unaffected by minor experimental adjustments. These findings confirm that the method is consistent and dependable in practical laboratory settings.

5. Discussion

A simple, rapid, and dependable UV spectrophotometric method based on the Area Under the Curve approach was successfully developed and validated for the quantitative estimation of silibinin. The method was designed using a Quality by Design framework and further refined through the Central Composite Design, which enabled systematic evaluation and optimization of all critical analytical variables. The finalized method exhibited excellent linearity, with an R squared value of 0.999, along with strong sensitivity and precision throughout the concentration range of 2 to 16 micrograms per millilitre.

Comprehensive validation carried out according to the International Council for Harmonisation Q2 R1 guidelines confirmed that the method meets all essential requirements of accuracy, reproducibility, and robustness. The percent relative standard deviation values remained consistently below two percent for all validation studies, reflecting stable and reliable analytical performance. The low limit of detection of 3.975 micrograms per millilitre and the low limit of quantification of 12.046 micrograms per millilitre further demonstrated the suitability of the method for routine quantitative assessment of silibinin.

Overall, the developed UV Area Under the Curve method is straightforward, cost effective, and highly dependable. These attributes make it a valuable analytical tool for routine quality control and standard evaluation of silibinin in both pharmaceutical formulations and herbal products.

Conflict of interest

The authors have no conflicts of interest regarding this investigation.

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