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Chromatographic Assurance: Validating Analytical Methods for Novel N-(4-Bromophenyl)-2-Phenyl-6,7-Dihydro-5hCyclopenta[D]Pyrimidin-4-Amine Using Rp-Hplc by a Qbd Approach

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KEYWORDS

HPLC-UV, Method validation, Quantification, ICH guidelines

ABSTRACT:

Introduction: A novel, fast, economical, and reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) technique was developed for determining N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine in bulk drug substance.

Objectives: The study was to design to develop a chromatographic method for the estimation of N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine which is simple, precise, accurate, and cost-effective N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine by RP-HPLC and validate as per ICH guideline. (ICH Q2 (R1)

Methods: During the experiment, the method was found trustworthy in eluting drugs using an isocratic approach on a C18 column (4.6 mm x 250 mm, 5.0 µm) at room temperature, employing a mobile phase composed of methanol and water in a ratio of 80:20, flowing at 1 mL/min, with U.V. detection set at 249 nm.

Results: The developed method was validated and displayed linear behavior within a concentration range of 10-50 µg/mL (r2=0.999), with a detection limit of 0.017 µg/ml. Method accuracy ranged from 99.95% to 100.37%. Intra-day and inter-day precision fell within the range of 0.13-0.96 % to 0.11-0.63 %, respectively. This validated method, demonstrating exceptional selectivity, Linearity, sensitivity, Precision, and accuracy, is suitable for quantifying the model drug in both bulk drug substances and pharmaceutical formulations.

Conclusions: The method was found to be reliable and robust, which was confirmed through QbD analysis. No experiments have been performed and documented to till date for N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine by HPLC.

1. Introduction:

N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine is a novel drug designed and synthesis for treatment of glioblastoma and the molecule shows better percentage inhibition score 82.91 ± 0.54 and 45.55 ± 1.46 for 100 µM and 10 µM respectively in glioblastoma cell line [1]. It is an organic compound belonging to the class of heterocyclic aromatic amines. The compound consists of a cyclopenta[d]pyrimidine core, a bicyclic structure incorporating a cyclopentane and a pyrimidine ring. This scaffold is further functionalized with bromophenyl and phenyl groups, which may impart unique chemical and biological properties to the molecule. The rigid and planar bicyclic structure with the bromophenyl group shows potential interaction with D.N.A., which is responsible for cancer cell proliferation [2]. In the present study, we have performed method development

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and validation using the QbD approach for the first time for N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine.

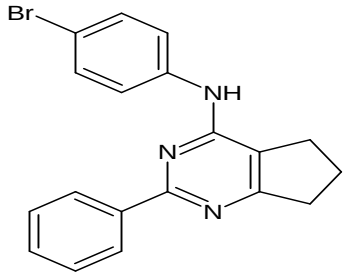

Figure 1: Structure of N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine

2. Materials & Methods:

The drug N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine is designed and synthesised in the laboratory. All solvents used for analysis were of analytical grade, including HPLC-grade methanol purchased from Sigma Aldrich (Mumbai), and HPLC-grade purified water was procured from J.K. Labs (Mumbai).

2.1 Instrumentation:

HPLC of Model Thermo Fisher Ultimate 3000, Thermo Fisher Scientific (U.S.A.). The injector port of the Thermo Fisher Ultimate 3000 HPLC system is a sophisticated component that ensures precise and accurate sample introduction. With features like an autosampler, temperature control, and seamless software integration, it enhances the efficiency and reliability of HPLC analyses. The injection process is controlled by Chromeleon Chromatography Data System (C.D.S.) software, allowing for automated, programmable injection sequences, method development, and data analysis. Chromatographic analysis was conducted using an Inertsil ODS-3V C18 column (4.6 mm x 250 mm, 5.0 µm). An ultraviolet (U.V.) spectrophotometer of the Shimadzu UV-1800 model was used to determine lambda maximum absorbance.

2.1.1 Preparation of reference standard solution:

A standard stock solution of the drug (1000 µg/mL) was prepared by weighing 10mg of the drug and transferring it to a 10 ml volumetric flask, and the volume was made up to 10 ml with methanol to get a concentration of 1000 µg/ml. To get 100 µg/ml, sufficient amounts of this solution were further diluted with methanol. Using the mobile Phase, this solution was further diluted to achieve final concentrations in the range of 1 to 50 µg/ml.

2.1.2 Selection of detection wavelength:

A standard solution with a concentration of 10 µg/ml was analyzed using an ultraviolet (U.V.) spectrophotometer at, scanning wavelengths ranging from 200 to 800 nm. The λ_{max} value was determined from the U.V. spectrum of the standard solution.

2.1.3 Selection of Mobile Phase:

Different trials were attempted on various mobile phases performed with varying ratios of water, (Acetonitrile) A.C.N., water-phosphate buffer, and finally, water-methanol. A good resolved and reproducible peaks were found in water-methanol with a ratio of 80:20 (v/v); hence, it was selected as the mobile Phase, followed by filtration and degassing through a 0.45 µm membrane filter before application. The mobile phase was then pumped through the system at a flow rate of 1 mL/min. The column temperature was maintained at 30°C throughout the analysis. The total runtime under these conditions was 10 minutes.

3. Analytical method validation:

Recorded evidence that offers a high level of assurance for a particular method that the procedure used to verify the analytical procedure is appropriate for its intended purpose is known as method validation. According to ICH Q2 (R1) requirements, the HPLC technique that was devised for model drug estimation was verified [6].

3.1 Linearity:

The working standard solution of 100 µg/mL was prepared from 1000 µg/mL stock solution using methanol and further diluted to obtain in the range of 10 – 50 µg/ml. The final solution samples were injected in HPLC. The drug's peak areas were plotted against the corresponding concentrations to create the calibration curves. The calibration curve's Y-intercept and Slope were calculated [7,8].

3.2 Precision:

Intraday and interday studies analyzed the Precision of the method. Three different concentrations (1, 20, and 40 µg/ml) of the drug were examined three times: once within the same day with a 3-hour interval and once on three separate days to determine intraday and interday Precision, respectively. The acceptable limit for % R.S.D. was set at less than 2.

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3.3 Accuracy:

The accuracy of an analytical method was determined by applying the method to analyze samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay. To test the accuracy of the developed method, the percentage recovery of the drug was calculated at three different levels (80%, 100%, and 120%), using the standard addition method. The sample was prepared in triplicate and analyzed at a wavelength of 249 nm [10,11].

3.4 Robustness:

3.4.1 Robustness Using Conventional Analytical Process:

To determine the Robustness of the developed method, the experimental condition was purposely altered, and products were evaluated. In addition, the Robustness of the method was investigated under a variety of conditions, including changes in flow rate and detector wavelength. To study the effect of flow rate and wavelength on the resolution. The flow rate of the mobile phase was 1ml/min. To study the effect of flow rate on the resolution, 0.2 units changed it from 0.8 to 1.2 ml/min, while the other mobile phase component was held as stated in chromatographic conditions. The wavelength λ max was 249 nm for the drug. It was changed to 247nm and 251nm while the other mobile phase components were held constant, as stated in the chromatographic condition [12].

3.4.1.1 Robustness using A QbD-based Central Composite Design:

A QbD approach employed a Central Composite Design with three levels, incorporating 6 axial points, 6 centre points, and 8 factorial points, a total of 20 batches as suggested by Design Expert software version 13. Factors such as wavelength, % organic content, and flow rate were considered, with target responses being peak height, peak area, and symmetry. Utilizing Design Expert software, as indicated in Table I. The percentage contribution of each factor was predicted, followed by ANOVA statistical analysis. Additionally, 3D response surface plots, contour plots, and overlay plots were generated for graphical representation and optimization of the method [13].

3.5 Limit of detection (L.O.D.):

It is the lowest amount of analyte in a sample that can be detected, but it is unnecessary to constitute under the stated experimental conditions. The detection limit was calculated using the following equation per ICH guidelines.

The L.O.D. was determined by using the following formula:

$$LOD = 3.3\frac{\sigma}{s}$$

Where,

σ = Standard deviation of the response and

S = Slope of the corresponding calibration curve.

3.6 Limit of quantification (L.O.Q.):

It is the lowest concentration of an analyte in a sample that can be determined with acceptable Precision and accuracy under stated experimental conditions. The quantification limit is calculated using the following equation as per ICH guidelines. [13, 14]

The L.O.Q. was determined by using the following formula:

$$LOQ = 10\frac{\sigma}{s}$$

Where,

σ = Standard deviation of the response and

S = Slope of the corresponding calibration curve.

4. Result and Discussion:

4.1 Determination of λ_{max}

From the standard solution's U.V. spectrum, the wavelength corresponding to maximum absorbance (λ_{max}) was determined to be 249 nm, as depicted in Figure 2.

Table I: Central Composite design for robustness testing using factors and obtained responses

Runs	Factors			Responses		
	Flowrate (ml)	Wavelength (nm)	% Organic concentration	Area (%)	Height (cm)	Symmetry (cm)
1	0.8	247	1	92	8.6	0.891
2	0.8	247	40	3990	360.9	0.866
3	1	245.6	20.5	1838	168.6	0.877
4	0.8	252	40	3998	366.3	0.925
5	0.6	249	20.5	1842	172.3	0.877
6	1.2	251	1	92	8.6	0.891
7	1.3	249	20.5	1838	168.6	0.877
8	0.8	251	1	95	8.9	0.891
9	1.2	251	40	4001	400.2	0.866
10	1.2	247	1	98	8.9	0.893
11	1	249	20.5	1838	166.2	0.870
12	1.2	249	40	4023	402.2	0.869
13	1	249	50	4967	409	0.859
14	1	249	20.5	1838	169.3	0.883
15	1	249	20.5	1838	166.6	0.891
16	1	249	10	958	87.8	0.862
17	1	249	20.5	1838	167.5	0.869
18	1	249	20.5	1838	168.6	0.879
19	1	249	20.5	1838	170.2	0.899
20	1	252.36	20.5	1838	179.8	0.855

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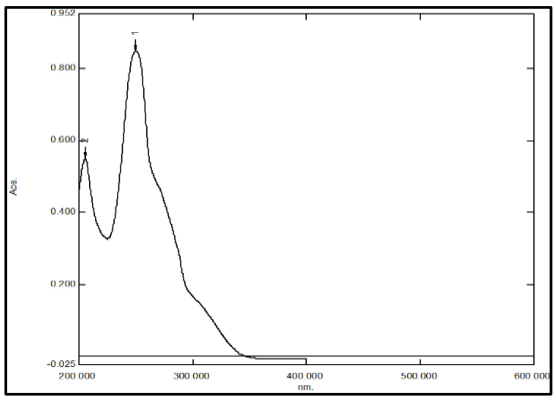

Figure 2: UV spectrum of N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine (10 ppm)

4.2 System Suitability Parameters for N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine:

To assess the system's suitability, a solution of 20 µg/mL was prepared by diluting a stock solution of 1000 µg/mL accordingly, as shown in Figure 3. System suitability parameters were evaluated by injecting the prepared standard solution six times and measuring various parameters, including theoretical plates, retention time, tailing factor/asymmetry, and % R.S.D. for RP-HPLC. As per ICH guidelines, the % R.S.D. should be below 2%, the asymmetry factor should be less than 2, and the theoretical plates (indicative of the column's separation efficiency) should exceed 2000. Tables II & III indicated System suitability parameters and optimised chromatographic conditions.

Table II: A summary of system suitability test parameter

HPLC system Parameters	HPLC assay
Concentration	20 µg/ml
R.T. (min)	6.9 min
Area	1838.66 ± 11.84
Theoretical Plates	More than 2000
Asymmetry	0.8

Table III: Optimized Chromatographic conditions

HPLC system parameters	Conditions
Column	Inertsil ODS-3V C18 column (4.6 mm x 250 mm, 5.0 µm)
Mobile Phase and diluents	Methanol: Water in the ratio of 80:20 v/v
Flow rate	1.0 ml/min
Detection (λmax)	**249 nm**
Detector	Ultraviolet (UV) detector
Temperature	30ºC
Injection volume	20 µl
Retention time	6.9 min
Run time	10 min

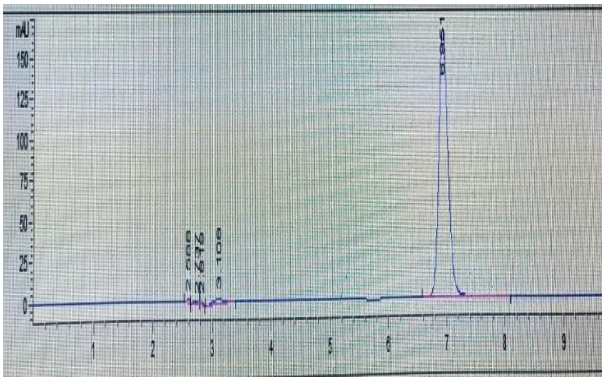

Figure 3: 20 ppm system suitability peak of the N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine

4.3 Method development:

4.3.1 Linearity:

The N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine is a drug stock standard solution was appropriately diluted with deionized water to prepare standard solutions ranging from 1 to 50 µg/ml. Each standard solution underwent three injections into the HPLC system under the specified chromatographic conditions. The Linearity of the method was assessed across six concentration levels within the 1–50 µg/ml range using regression analysis. The calibration curve

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was generated by plotting the average peak area against the standard concentration, as shown in Figure 3. The correlation coefficient, Slope, and intercept of the regression line were determined using the least squares method. The relationship between the mean peak area (Y, n=3) and concentration (X) was found to be linear, represented by the equation Y = a + Bx. The values obtained for Slope, intercept, and correlation coefficient ® were 100.31, 56.14, and 0.9991, respectively, as presented in Table IV and V.

Table IV: Linearity study of N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine

Conc. (µg/ml)	Avg Area	SD	% R.S.D.
1	92.000	1.000	1.087
10	958.333	7.638	0.797
20	1838.667	11.846	0.644
30	2963.333	35.529	1.199
40	3990.667	33.858	0.848
50	4967.333	52.918	1.065

Table V: Calibration parameters

Parameter	Result
Conc.	10-50 µg/ml
Slope	100.31
Intercept	56.14
Correlation coefficient	0.9991

4.3.2 Precision:

The % R.S.D. for three repeated measurements of the same concentration (1, 20, 30, µg/ml), was observed to be below 0.096. Table VI presents the interday and intraday precisions. A % R.S.D. value below 2 indicates the Precision of the developed method.

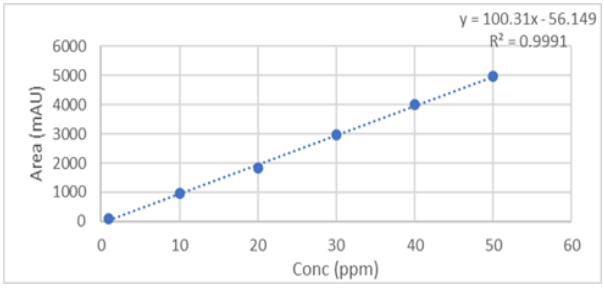

Figure 3: Standard calibration curve of N-(4-bromophenyl)-2-phenyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine (1-50 µg/ml)

Table VI: Intraday and Interday precision

Sr. No.	Precision Period	Parameter	Conc. (µg/ml)	Mean area (n=3)	S.D.	% R.S.D.
1	Intraday Precision	Morning	1	91.500	0.500	0.546
			20	1857.000	13.748	0.740
			40	4003.667	9.074	0.227
		Afternoon	1	90.767	0.751	0.827
			20	1865.000	10.000	0.536
			40	4014.667	13.051	0.325
		Evening	1	90.667	0.511	0.563
			20	1863.333	18.009	0.967
			40	4028.667	5.508	0.137
2	Interday Precision	Day-1	1	90.800	0.265	0.291
			20	1854.667	11.676	0.630
			40	4061.333	14.572	0.359
		Day-2	1	90.767	0.252	0.277
			20	1843.333	6.658	0.361
			40	852.900	1.015	0.119
		Day-3	1	91.500	0.500	0.546
			20	1889.333	5.132	0.272
			40	4087.000	10.149	0.248

4.3.3 Accuracy:

A predetermined quantity of standard solution was introduced into the sample solutions, previously examined at three distinct levels (80%, 100%, and 120%). The amount of drug recovered was determined for each concentration. The recovery results are presented in Table VII. Percent recovery was found to be 99.5 to 100.37 in range, suggesting that pharmaceutical formulation excipients are free from any interference and confirming the high accuracy of the analytical method.

4.3.4 Robustness studies:

4.3.4.1 Robustness Using Conventional Analytical Process:

The findings indicated the minimal influence of alterations in mobile phase flow rate and detector

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wavelength on the chromatographic characteristics of the drug. Adjustments in these parameters had only a slight effect on the retention time of the drug. The study results, depicted as % Relative Standard Deviation (R.S.D.), were showcased in Table VIII.

Table VII: Accuracy study

Sr. No.	Spike level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	80	32	31.98	99.94	99.95
	80	32	31.94	99.82	
	80	32	32.02	100.09	
2	100	40	39.97	99.94	100.00
	100	40	40.09	100.23	
	100	40	39.93	99.83	
3	120	48	48.20	100.43	100.37
	120	48	48.18	100.39	
	120	48	48.14	100.30	

Table VIII: Robustness using conventional analytical method

Condition	Variation	Conc.	Average	SD	RSD (%)
Mobile Phase flow rate	0.8 ml/min	1 ppm	71.567	0.404	0.565
		20 ppm	1471.667	15.275	1.038
		40 ppm	3271.667	15.822	0.484
	1.2 ml/min	1 ppm	111.600	0.361	0.323
		20 ppm	2264.667	7.572	0.334
		40 ppm	4854.333	60.707	1.251
Change in wavelength	247 nm	1 ppm	90.833	0.764	0.841
		20 ppm	1881.667	15.275	0.812
		40 ppm	4008.333	20.817	0.519
	249 nm	1 ppm	90.933	0.404	0.444
		20 ppm	1806.000	8.544	0.473
		40 ppm	4069.333	13.317	0.327
	251 nm	1 ppm	90.233	0.751	0.832
		20 ppm	1794.667	13.429	0.748
		40 ppm	4020.333	18.175	0.452

4.3.4.2 Robustness using A QbD-based Central Composite Design:

During validation studies, chromatographic parameters such as peak area, peak height, and symmetry were adjusted, all the analyses were adequately resolved, and the elution order remained unchanged. This means that the chromatographic conditions were optimised to achieve the analyte's desired separation. The selected factors viz. peak area and retention time remained unaffected after making variations in the flow rate, composition of mobile Phase, and pH of mobile Phase. Flow rate should be ± 0.1, pH of mobile phase ± 0.2, composition of mobile Phase ± 2%Acceptable. % R.S.D. values obtained after making small deliberate changes in the method indicate that the method is robust for the intended purpose. AQbD approach C.C.D. design was employed to perform a robustness study which was indicated in Table I. For studying cause and effect. The Ishikawa fishbone diagram provides a structured approach to identifying and analyzing the root causes of a problem, facilitating effective problem-solving and decision-making processes within organizations as indicated [4]in Figure 4. Factors or critical method parameters (C.M.P.s) were selected such as wavelength, % organic, and flow rate at three levels and Four Critical Quality Attributes (CQAs) taken were area, height and symmetry. 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 are significant differences in their values Table IX. Equations obtained from the model were as:

AreaY_1 =+1847.26 +2.36A - 1.24 B+1924.16 C-4.87AB+4.12 AC- 1.38 BC+ 13.05 A^2 +12.34 B^2 + 144.75 C^2……**equation (1)**

Height Y_2 = +179.29+5.12A+1.55B+178.89C……

……**equation (2)**

SymmetryY_3 = -3.77+4.99A+0.018659 B – 0.0448C - 0.02AB-0.0018 AC+0.000186BC……...

……**equation (3)**

From the equations, a positive sign indicates a synergistic effect, and a negative sign indicates an antagonistic effect in the polynomial equation. From the Table of ANOVA, responses Y_1, Y_2, and Y_3 indicated that predicted values for all the factors are under satisfactory value. The quadratic method was suggested by software for all CQA for the area, Non-linear for concentration, and (2FI) for symmetry and was not significant, was suggested. P-values (less than 0.05) were significant for all responses.

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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. 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 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-axis, 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 of factors to achieve a desired outcome. It also helps in interpreting interactions between factors. Patterns in the 3D plot can indicate whether factors interact synergistically, antagonistically, or independently.

Overall, both 3D plots and contour plots in Design Expert software are essential tools 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 ,6 ,7.

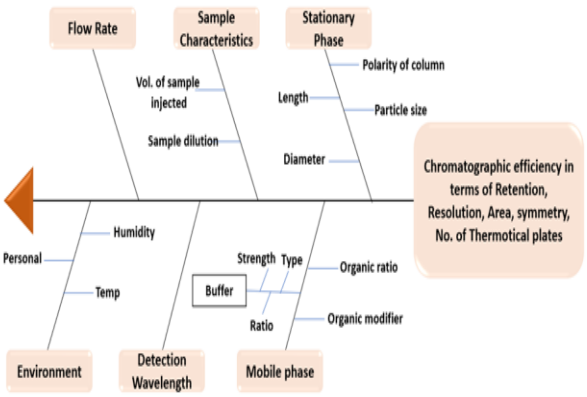
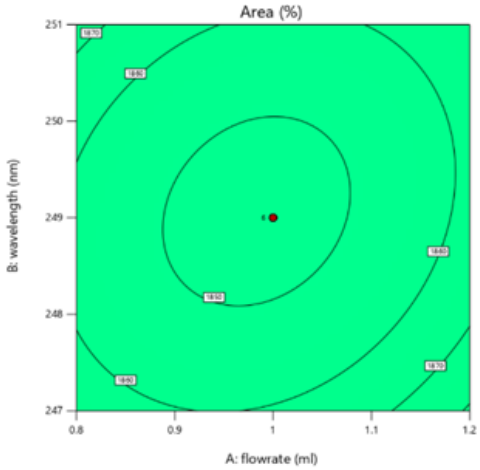


Figure 4: Ishikawa fishbone diagram showing causes and effects

Table IX. Statistical parameters by ANOVA analysis for the responses

Parameters	S.S.	df	MS	F-value	p-value	Model F-value	Model p-value	Prob > F
Response Y_1 (Area)								
Flowrate (A)	0.0010	1	76.26	0.0154	0.9038	910.65	0.0019	significant
Wavelength (B)	0.0001	1	21.16	0.0043	0.9492			
%Concentration(C)	0.0003	1	3.47	7007.12	0.0001			
Response Y_2 (Height)								
Flowrate	358.56	1	358.56	0.88	0.3613	276.98	0.0001	significant
Wavelength	33.02	1	33.02	0.0813	0.7792			
%Concentration	3.37	1	3.370	829.97	0.0001			
Response Y3 (Symmetry)								
Flowrate	0.0025	1	0.0002	1.50	0.2426	2.90	0.0507	Not significant
Wavelength	0.0002	1	0.0003	2.34	0.1502			
%Concentration	0.0003	1	0.0006	4.08	0.0645			



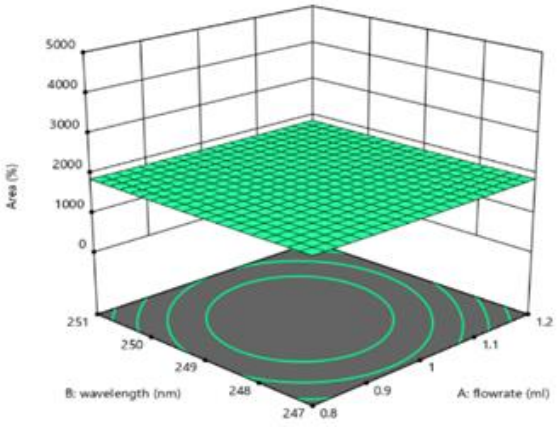


Figure 5: Contour plot and 3-D plot of response peak area in contrast to wavelength and flow rate

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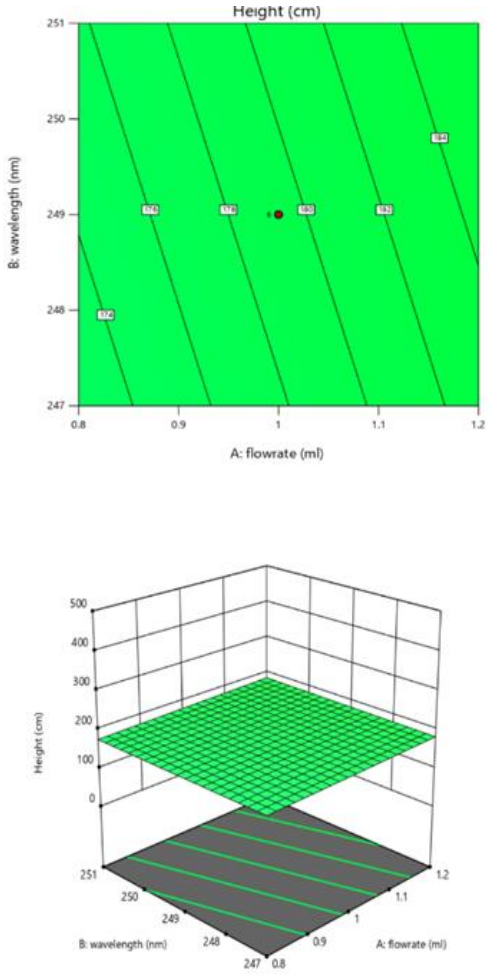
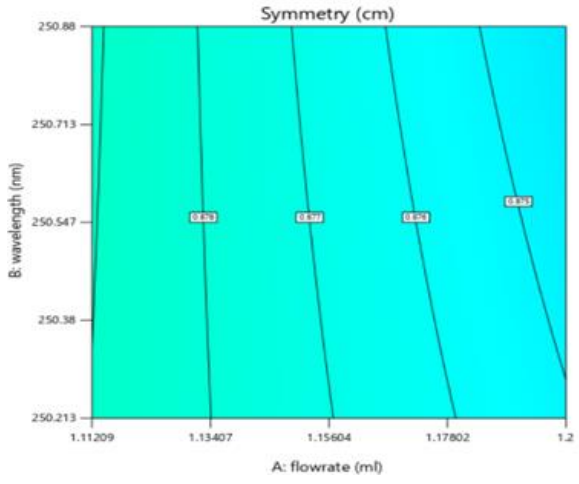


Figure 6: Contour plot and 3-D plot of response height in contrast to wavelength and flow rate



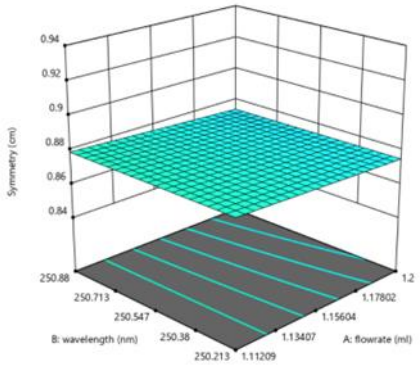


Figure 7: Contour plot and 3-D plot of response Symmetry in contrast to wavelength and flowrate

4.3.5 Limit of detection and Limit of quantitation:

The Limit of Detection (L.O.D.) and Limit of Quantitation (L.O.Q.) for the model drug, determined based on the standard deviation of the Slope and intercept, were observed to be 0.017 µg/ml and 0.053 µg/ml, respectively.

Table X. Summary of Validation Parameters by HPLC

Sr. No.	Validation parameters	Results
1.	Linearity equation R^2	y=100.31x-56.149
	Range	R^2 =0.9991
		1 to 50 µg/ml
2.	Precision	(% R.S.D.)
	Intraday	0.137 - 0.967
	Interday	0.119 - 0.630
3.	Accuracy	Mean ± %RSD
	80	99.95 ± 0.523
	100	100.00 ± 0.476
	120	100.37 ±0.609
4.	LOD	0.017 µg/ml
	LOQ	0.053 µg/ml,
5.	Specificity	Specific
6.	Robustness	Robust

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5. Conclusions:

The current study emphasizes the importance of creating and verifying a reverse-phase high-performance liquid chromatography (RP-HPLC) technique for the first time; no other documented reports have been observed until now to develop, validate, and analyze using the QbD approach. The RP-HPLC method was highly effective for isolating and identifying the quantity. As part of the validation process, the following parameters were evaluated: Linearity, accuracy, the limit of quantification (L.O.Q.), the limit of detection (L.O.D.), Precision, and the method found to be desirable and robust using the AQbD approach. This technique avoids lengthy chemical procedures, making it suitable for regular analysis.

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8. Conflicts of Interest:

The authors declared no conflict of interest.

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