GREENNESS-ASSESSED STABILITY-INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF CABOZANTINIB BY AQbD DRIVEN STRATEGY

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ABSTRACT

Objective: The study was conducted to develop, optimise, and validate a rapid RP-HPLC method for stability evaluation of Cabozantinib using AQbD principles integrated with green analytical chemistry assessment, providing a systematic and environmentally sustainable analytical approach.

Methods: Method optimization was performed using a custom design approach by assessing flow rate, organic solvent ratio, pH of solvent. A Waters C18 analytical column (150 × 4.6 mm, 5 µm) was performed under isocratic conditions that was used in the final optimized conditions. The mobile-phase consisted of phosphate buffer and methanol (60:40 v/v) ratio, with a pH adjusted to 4.6. Detection was achieved at 248 nm while maintaining a flow rate of 1.0 mL/min. Greenness was assessed using both AGREE and the MoGAPI tools.

Results: The optimized technique using custom design from JMP® Version 18 software yielded global desirability function of 77.6% for Cabozantinib. A Retention time of 2.80minutes was determined. Linearity range was of 50-150 µg/ml had an R^2 value is 1.LOD and LOQ values for the technique were 0.165 µg/mL and 0.550 µg/mL, respectively. The percentages of degradation under heat stress and acid stress were 9.53% and 11.95%, respectively. Greenness Assessment yielded an AGREE score of 0.8 and MoGAPI score of 80, reflecting greener analysis.

Conclusion: The proposed RP-HPLC method was rapid, reliable and stable, with adequate validation and environmental sustainability. The procedure is suitable for regular stability evaluations and quality monitoring.

Keywords: Design of Experiment, Custom Design, Greenness, AGREE, MoGAPI

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1. INTRODUCTION

Cabozantinib is a multitargeted tyrosine kinase inhibitor used to treat a variety of malignancies, including medullary thyroid carcinoma (MTC), hepatocellular carcinoma (HCC), and renal cell carcinoma (RCC). It works by suppressing receptor tyrosine kinases like MET, VEGFR, RET, and AXL, which are implicated in tumour growth, angiogenesis, and metastasis. Cabozantinib efficiently limits the proliferation and spreads of cancer cells by inhibiting various signaling pathways. The therapeutic significance, precise quantification is required for regulatory compliance and quality assurance.[1]

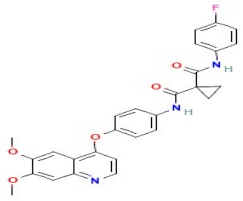


Figure 01. Structure of Cabozantinib

The study is required to ensure the method reliability, enhanced by understanding the critical method parameters, regulatory compliance and consistent performance through the product lifecycle. AQbD follows methodical, science-driven strategy for developing an analytical technique that focuses on established objectives, risk evaluation, determination of key method parameters, and construction of operating space through experimental design (DoE). This method enhances method understanding, robustness with lifecycle

management while minimizing variability and enhancing compliance with regulatory requirements.[2, 3]

Reverse-phase high-performance liquid chromatography is a versatile as well as reproducible method in pharmaceutical analysis.[4] Method development and validation ensure accuracy and robustness per ICH guidelines, while stability studies or force degradation verify its capable of assessing stability, thereby making it appropriate for routine analytical quality. Force degradation studies verify Cabozantinib's stability-indicating character, making it appropriate for routine quality assessment, while method development and validation ensure reliable and accuracy following ICH requirements.[5]

Although RP-HPLC methods for Cabozantinib have been reported, limited studies have integrated AQbD together with green analytical chemistry concept for analytical method development. The current work intended to design and verify a quick, stability-indicating RP-HPLC technique employing AQbD, as well as to analyse its environmental sustainability using the AGREE and MoGAPI evaluation tools.

2. MATERIALS AND METHOD

2.1 Materials

Cabozantinib reference standard was acquired from Rainbow Pharma Training Labs in Bengaluru, India. Methanol, hydrochloric acid, sodium hydroxide, hydrogen peroxide, potassium dihydrogen phosphate (KH_2PO_4), and HPLC-grade water was obtained from Thermo Fisher Scientific (India) Pvt. Ltd. in Mumbai city, India.

2.2 Instrumentation

Analysis was carried using a Waters Alliance 2695 HPLC system fitted with 2489 UV-Visible detector. Data acquisition and processing were performed using Empower 3 software. Statistical optimization was conducted using JMP® Version 18 software. Greenness was assessed using the AGREE and MoGAPI software tools.

2.3 Method Development

Preparation of buffer: 1.70 g of KH_2PO_4 was weighed and dissolved in water to prepare 250 mL of solution, filtered through 0.45-µm membrane filter followed by 10 minutes of sonication.

Preparation: The buffer solution was first degassed using an ultrasonicator for duration of 10 minutes. A solution was then prepared by combining 125 ml of the KH_2PO_4 buffer and 125 ml of methanol in a 1:1 ratio. Filtration through a 0.45-µm membrane filter was performed to further separate the resulting mixture after vacuum filtration.

Standard stock solutions: An accurately weighed 10 mg quantity of cabozantinib reference material was dissolved in methanol and the volume was adjusted to 25 ml. Therefore, 10 ml of HPLC-garde water was subsequently diluted with 1 ml of solution.

2.4 AQbD-Based Method development

The AQbD workflow consisted of defining the Analytical Target Profile (ATP) was defined, followed by the identification of Critical Method Attributes (CMAs) and Critical Method Parameters (CMPs). [6] A risk identification and assessment was performed using Ishikawa diagram as shown in figure 02. Flow rate, buffer strength , and column configuration were selected as independent variables, whereas chromatographic retention time, USP plate number and tailing value were selected as analytical response variables.[7] A custom design of experiment (DoE) was created with JMP® Version 18 software to assess the individual and interaction impact of these factors and determine the best chromatographic conditions.[8]

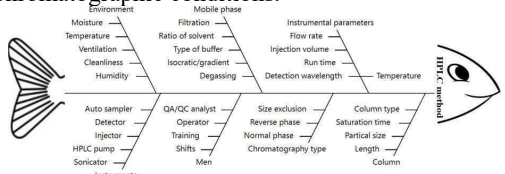


Figure 02. Ishikawa (fishbone) diagram illustrating risk assessment

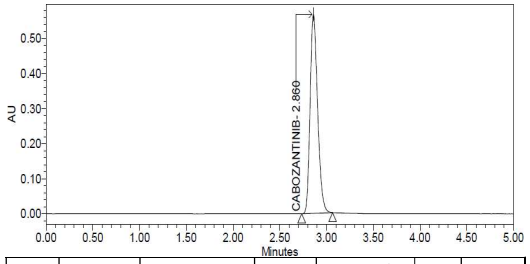
2.5 Optimized chromatographic condition: A Waters C18 analytical column (150 × 4.6 mm, 5 µm) was used to achieve optimal chromatographic separation. The phosphate buffer and methanol (60:40, v/v) eluent was supplied at a flow rate of 1.0 mL/min. The temperature of the column was kept at 25°C while UV detection was performed at a wavelength of 248 nm.[9]

2.6 Custom experimental design

The analytical method was optimized with a custom experimental design. Different ranges of higher and lower limits were chosen for rate of flow varying from 0.5 to 1 ml/min, buffer concentrations between 40-60, and column: GL Sciences and Waters. JMP® Version 18 software was used to develop and analyze a 12-run study design. As illustrated in Table 01.

Table 01. Method runs by Custom Design

Sl. no	Factors			Responses		
	Flow rate	Buffer Concentration	Column	Retention	USP Plate count	Tailing Factor



				ime		
1	0.5	60	GL Sciences	3.506	6291	1.31
2	0.5	40	Waters	3.532	7749	1.9
3	0.5	60	Waters	3.163	6597	1.41
4	1	40	Waters	2.924	8572	1.69
5	0.5	60	GL Sciences	3.503	5612	1.22
6	0.5	40	Waters	3.608	7948	1.92
7	1	60	Waters	2.824	6839	1.2
8	1	60	Waters	2.648	6679	1.23
9	0.5	40	GL Sciences	3.509	6871	1.64
10	1	40	GL Sciences	3.263	7364	1.48
11	1	40	GL Sciences	3.259	7285	1.46
12	0.75	50	GL Sciences	3.195	6439	1.37

2.7Analytical Method Validation

The RP-HPLC method underwent validated using ICH Q2(R2) guidelines covering system appropriateness, specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. Six replicate injections of the standard solution were performed to evaluate the suitability of the system. Specificity was evaluated by comparing blank, standard along with stressed sample chromatograms. The linear response was demonstrated over concentration range of 50-150 µg/mL. The standard addition method was used to determine accuracy at concentrations of 50%, 100%, and 150%, while precision was assessed using six replicate injections at the 100% test concentration. The LOD and LOQ were determined based on the signal-to-noise ratio method in compliance with ICH guidelines. Robustness was evaluated by intentionally varying chromatographic settings to determine the dependability and consistency of the established approach. [10, 11]

2.8 Forced degradation studies

Studies on forced deterioration were carried out in oxidative, alkaline, and acidic, hydrolytic, thermal, and photolytic stress circumstances to examine the stability evaluation capability of proposed analytical method. Stressed samples were analysed using the optimized chromatographic parameters, and the obtained degradation profiles were compared against the untreated sample. [12, 13]

2.9 Green assessment

AGREE is a software-based greenness assessment tool that quantitatively evaluates the analytical method using 12 principles of Green analytical chemistry. MoGAPI (Modernized Green Analytica procedure Index) is an advanced greenness assessment tool which expands from original GAPI tool which provides more detailed evaluation zones, better colour gradation and enhanced ability to differentiate different methods involved from sample collection to waste disposal. [14, 15]

3 RESULTS AND DISCUSSION

3.1 Method Development: A Waters C18 analytical column having dimensions of 150 × 4.6 mm with 5 µm containing KH_2PO_4 combined with methanol in ratio 60:40 (v/v) in 1.0 mL/min and monitored at 248 nm yielded retention time of 2.860 minutes under the ideal conditions, as illustrated in figure 03.

Figure 03. Chromatogram of the optimized method (standard chromatogram)

3.2 Analytical Quality by Design

Effect of Critical Process Parameters

Statistical analysis demonstrated that flow rate, buffer concentration, and column type significantly influenced the chromatographic outcomes, including chromatographic retention time, plate count, along with the tailing factor. These key method parameters were therefore chosen for optimization using the AQbD approach.

Numerical Optimization and Prediction Profiler

As seen in figure 04, which displays a global desirability value of 77.69%, numerical optimization indicates that the predictive profile analysis provides a consistent relationship between the input parameters and the measured responses, showing fluctuations at extreme response values. This suggests that the model is valid for accomplishing desired goals for all three responses.

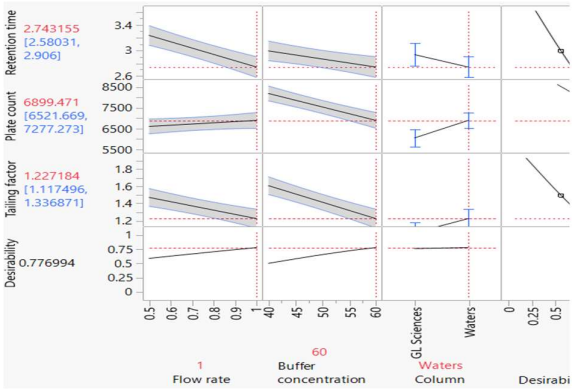


Figure 04. Prediction profiler with maximized desirability

Model Adequacy and Statistical Analysis

The regression models showed excellent agreement between the experimental and predicted responses. The obtained R^2 values (>0.90) and statistically significant p-values (<0.05) confirmed that the developed models adequately described the influence of the selected method variables on the chromatographic performance.

3.3 Analytical Method Validation

System suitability: System suitability evaluation results demonstrated method reliability: the obtained tailing factor was found to be 1.14 (<2.0), plate count 6,985 (>2,500), retention time stable at 2.860 minutes with acceptable peak area, and %RSD only 0.1% (≤2%), all meeting required limits Table 02.

Table 02: System suitability

Sl.no	Sample name	Analyte	Retention time (min)	Peak Area (µV.sec)	Theoretical plate count	Peak tailing factor
1.	Standard-2	Cabozantinib	2.858	3186270	6888	1.26
2.	Standard-2	Cabozantinib	2.854	3167385	6810	1.26
3.	Standard-2	Cabozantinib	2.852	3175173	6799	1.27
4.	Standard-2	Cabozantinib	2.849	3168360	6843	1.27
5.	Standard-2	Cabozantinib	2.849	3172060	6855	1.27
Mean				3173849.6		
%RSD				0.2		

Accuracy: Accuracy was evaluated at concentrations of 50%, 100%, and 150%, and recovery percentages were computed and results Yielded 99-100% recoveries, meeting ICH guidelines specification limit in 98-102%, the results as shown in Table 03.

Table 03. Accuracy

Parameters	Trail (50%)	Trail (100%)	Trail (150%)
Retention time	2.841	2.846	2.853
	2.841	2.849	2.855
	2.839	2.847	2.854
Area	1564919	3156975	4711454
	1563408	3156382	4711048
	1574661	3165092	4712844

Precision: The precision of a system and technique was determined by examining the data concordance between series of measurements. Six injections of 100% concentration were administered in this case. With a precision %RSD of 0.1%, the proposed method provided a high level of precision as results are shown in Table 04.

Table 04. Precision

Sl No.	Sample Name	RT	Area
01	PRECESION-1	2.851	3153623
02	PRECESION-2	2.850	3152649
03	PRECESION-3	2.847	3161263
04	PRECESION-4	2.849	3150696
05	PRECESION-5	2.860	3156699
06	PRECESION-6	2.847	3167397

Specificity: By examining the effects of blanks and other contaminants during the cabozantinib retention period, the specificity of the suggested method was established. As a result, no new peak was identified in the blank, indicating a high level of specificity for the proposed approach.

Detection and Quantification Limit: The LOD and LOQ was determined based on the (S/N) ratio, which resulted at 3.8 and 10.1, respectively. The calculated detection limit (LOD) was determined as 0.165 µg/mL. The minimum sample quantity for quantification was found to be 0.550µg/ml (LOQ).

Linearity and Range: Cabozantinib solution (50–150 µg/ml) was injected at 248nm wavelength. The graph shows that R^2 was found to be 1, indicating that Cabozantinib exhibits linearity over the 50% to 150% range, shown in figure 05.

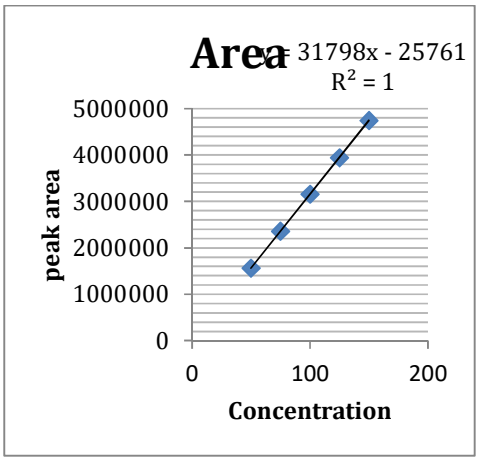


Figure 05. Linearity of Cabozantinib

Robustness: The outcomes met system suitability requirements, confirming that the development method was robust and exhibited no notable changes when these parameters were slightly altered.

3.4 Forced Degradation Studies

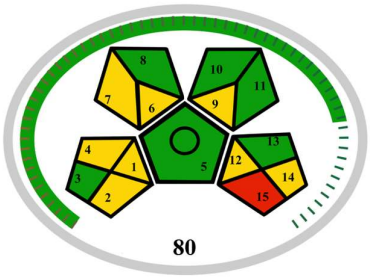
Extensive stress testing of cabozantinib was conducted under a range of conditions, such as high temperatures, photolysis, acidic and alkaline environments, oxidative stress, and exposure to water. With distinct but comparable degradation peaks, Table 05 summarizes the notable degradation that was observed, particularly under heat and acidic stress, % degradation was found to be under the ICH acceptable limit.

Table 05. Forced degradation study

Stress	Sample weight (mg)	Sample Area	% Assay	%Degraded
Acid stress	350	2800225	88.05	11.95
Base stress	350	2916317	91.70	8.93
Peroxide stress	350	2996560	94.23	5.77
Heat stress	350	2877043	90.47	9.53
Photo stress	350	2980642	93.72	6.28
Hydrolysis stress	350	3142857	98.83	1.17

3.5 Evaluation of Greenness

The MoGAPI index and the AGREE tool, provide thorough evaluation based on 12 Analytical Green Chemistry principles used to assess method greenness. The MoGAPI tool overall score was 80 as shown in the figure 06, while AGREE tool yielded a score of 0.8 as shown in figure 07 both demonstrating that the method possesses high degree of environmental compatibility as well as highly acceptable from the environment.



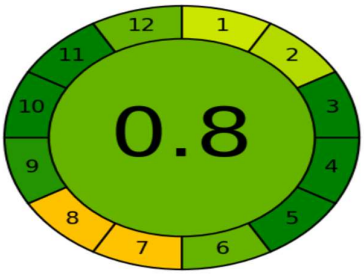


Figure 06. MoGAPI Score **Figure07.AGREE Score**

4. CONCLUSION

The AQbD guided RP- HPLC method development for Cabozantinib demonstrated enhanced accuracy, precision, sensitivity and robustness via custom design whose multiple regression analysis exhibited significant effects of critical method attributes, yielding optimized method conditions. Method validation was performed based on the validation criteria established by ICH guidelines, with all validation outcomes meeting acceptance criteria, while the model fit was confirmed by the close match between expected and experimental responses. Stability indicating forced-degradation studies displayed non interfering degradants peak. AGREE and MoGAPI based greenness assessment confirms sustainability and environmentally favourable HPLC procedure compared with available existing literature based on RP-HPLC method development. This is appropriate for routine quality evaluation of Cabozantinib across industry as well as research use.

5. Conflict of Interest: The authors declare no conflict of interest.

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