RESEARCH ARTICLE

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF AMIVANTAMAB USING A QUALITY BY DESIGN APPROACH

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ABSTRACT

The current research describes the development and validation of a robust stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for estimating Amivantamab, a bispecific antibody used to treat non-small cell lung cancer, using the Quality by Design approach. A custom design strategy using JMP® software was employed to discover and optimize critical method parameters (CMPs), including column selection, solvent composition, and flow rate. Chromatographic separation was achieved on a GI Sciences C8 column (150 × 4.6 mm, 5 µm) under isocratic conditions using mobile phase KH_2PO_4 buffer and methanol (80:20 v/v) at pH 4.2. The mobile phase was delivered at a flow rate of 1.0 mL min^{-1}, and analyte detection was performed at 248 nm using a UV–Visible detector. The retention time (RT) of Amivantamab was found to be 1.979 minutes. The method demonstrated acceptable linearity in the concentration range of 50-150 µg/ml with a correlation coefficient (R^2) of 1. The LOD and LOQ were found to be 0.013 µg/mL and 0.042 µg/mL, respectively. The developed RP-HPLC method was optimized by a Quality by Design approach and successfully validated in harmony with ICH guidelines. It is appropriate for routine quantitative assessment of Amivantamab in bulk form.

Key words: RP-HPLC, Amivantamab, custom design, QbD, Risk Assessment, Critical quality attributes, MODR.

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INTRODUCTION

Amivantamab is a bispecific monoclonal antibody that targets epidermal growth factor receptor (EGFR) and mesenchymal–epithelial transition (MET) receptors., and It is used for the treatment of adults with metastatic non-small cell lung cancer harboring EGFR mutations[1]. Amivantamab

is a human monoclonal antibody that utilizes Genmab's DuoBody technology to achieve bispecific binding. The drug has two functional arms: one arm binds to the interstitial area of the epidermal growth factor receptor (EGFR), preventing it from interacting with its ligand, the epidermal growth factor (EGF). Additionally, it is shaped with reduced levels of fucose in the Fc region, enhancing its ability to bind to immune effector cells and endorsing antitumor immune activity[2].

Biopharmaceuticals such as monoclonal antibodies involve rigorous analytical methods to ensure their quality, potency and safety[3]. Amivantamab, a therapeutic antibody, is susceptible to degradation when exposed to environmental and chemical stressors, such as heat, pH extremes, oxidative agents and light. Stability profiling and consistent quantification are essential to supporting its development, regulatory compliance and clinical use[4]. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) is generally employed for analyzing monoclonal antibodies and similar products due to its excellent resolution, sensitivity, and specificity[5-7].

Quality by Design (QbD) is a structured developmental methodology that initiates with clearly defined ideas and emphases on product and process understanding, along with process control guided by quality risk management[8]. QbD focuses on designing and optimizing manufacturing and formulations processes to consistently meet predetermined product specifications, making it a vital component of modern pharmaceutical development[9]. In the field of analytical chemistry, in the 21st century Analytical Quality by Design (AQbD) was launched by the FDA's cGMP and improved by the ICH recommendations Q8 (R2). AQbD has emerged as a crucial prototype that is revolutionizing the development and validation of methods. In order to certify the standards and validity of the analytical techniques, this novel approach incorporates the concepts of QbD into analytical processes[10]. This approach focuses on ensuring quality in the process strategy rather than solely challenging the final results of the process[11]. U.S. FDA and the EMA regulate consistently promote the application of QbD principles to maintain product quality and ensure safety. It highlights the significance of assessing the quality of the analytical process during development[12].

Although Amivantamab holds significant therapeutic importance, limited research is available on the development of a validated RP-HPLC method employing the QbD approach. The present study intends to fill this gap by designing, optimizing and validating a robust RP-HPLC method for the quantitative assessment of Amivantamab.

METHODOLOGY:

Materials and Methods

Chemicals and Reagent

Amivantamab was procured from Sunlight Sciences, Hyderabad, India. HPLC-grade methanol, hydrogen peroxide, and acetonitrile were obtained from Honeywell, New Delhi, India. HPLC water was procured from Molychem Chemicals, Mumbai, India. KH_2PO_4, from Qualigens, Mumbai, India. NaH_2PO_4, HCl, and NaOH were purchased from Thermofischer, India[13].

Instrumentation and software

The research was conducted using a Waters Alliance HPLC 2695 system, equipped with C8 and C18 reverse-phase columns (GL sciences, size: 150×4.60 mm, 5 µm particle size) and a UV/Visible detector (Model 2489), operated using Empower 3 software was used for method development and validation. Method optimization was further supported through Custom Design in JMP Pro 14[14].

Preparation of Solutions

KH_2PO_4 buffer (0.1 M) preparation

Accurately weighed 1.70 g of KH_2PO_4 was transferred into 1000 ml volumetric flask, dissolved it in water, and volume was made. The solution was then filtered through a 0.45 µm membrane filter and sonicated for 10 minutes[15].

Preparation of Mobile phase

The mobile phase was prepared by mixing 500 ml of KH_2PO_4 buffer (50%) with 500 ml of methanol (50%). The mixture is then vacuum-filtered through a 0.45 µm membrane filter and degassed by ultrasonication for 10 minutes. The same mobile phase was also used as the diluent in this study[16].

Preparation of standard stock solutions

A precisely measured amount of Amivantamab (10 mg) was placed into a 10 ml volumetric flask, diluted with the mobile phase, and subjected to ultrasonication for 5 minutes to get a stock solution with a final concentration of 1000 µg/ml[17].

DEVELOPMENT OF METHOD

Quality Target Product Profile (QTPP)

The QTPP plays a significant role for recognising the variables that influence the critical method attribute constraints. For the proposed HPLC method, the QTPP includes accuracy, specificity, precision, LOQ, LOD, linearity, robustness, run time and system suitability (Table 1) [18].

Critical Quality Attributes (CQAs)

The CQAs are the process factors that are reliably affect the QTPP. Theoretical plate, retention time, tailing factor, flow rate, column temperature, and peak area are controlling critical method parameters necessary to preserve the QTPP's acceptable response range (Table 2).

Critical Method Attributes (CMAs) and Critical Performance Attributes

Risk Assessment:

Risk assessment is the systematic process that identifies potential risks to product quality by evaluation of critical material attributes and process parameters which affects the

CQAs, with the help of Risk Estimation Matrix table (REM) the potential risk factors affecting the product Critical Analytical Attributes (CAAs) were recognized [19].

Experimental design

The statistical experimental design called Custom design was used for the development of method runs. The various method parameters recognized with the help of risk assessment were incorporated in the experimental design (Table 4). The Custom design was devised by using JMP PRO 14 software (academic license; SAS Institute Inc., Cary, NC, USA). The study design generated a total of eight runs. For each method run, the response was obtained and incorporated into the design to analyse the statistical significance of model fit [20].

Optimal Method development: Optimized chromatographic conditions

The statistical desirability approach called Prediction profilers was used for the method optimization. In statistical optimization, desirability refers to a technique that transforms multiple objectives or constraints into single desirability value. This transformation allows for easier comparison and decision making among different method variables. The optimal chromatographic condition obtained as per the prediction profiler are presented in the Table 5. The Plate count, Peak area, tailing factor and Retention time was checked and confirmed for the optimized RP-HPLC method.

AQbD Method Validation: It is a crucial process in the pharmaceutical field to assure that a particular technique is appropriate according to its original purpose and produces reliable and accurate results. It involves a series of tests and assessments to confirm the performance, reliability and robustness of the analytical method[20].

Linearity and Range: Linearity of Amivantamab was assessed in the concentration range of 50-150µg/ml by preparing calibration standards and analyzing them using the optimized chromatographic conditions.

Precision: A 5 ml portion of the stock solution was pipetted and diluted to get a final concentration of 100µg/ml. six vials were prepared and injected, with the acceptance criteria set at 97-103% recovery and a relative standard deviation (RSD) not beyond 2% across all replicates.

Accuracy: To verify accuracy, the drug was spiked into samples at concentrations of 50%, 100%, and 150%, and calculating percentage recovery through comparison of the measured values with the corresponding expected amounts [21].

Robustness: The robustness of the system was investigated by adjusting its pH (±0.2), temperature (± 2 ◦C), flow rate (± 10 ◦C), and mobile phase composition (±5%) [22].

System suitability: System suitability was evaluated by injecting replication standard solutions before sample analysis. Theoretical plates (N), peak area, tailing factor and retention time were among the parameters evaluated. The approach was deemed appropriate if the theoretical plate count was ≥ 2500, the tailing factor was within acceptable bounds (≤2.0), and the RSD of peak area and RT was ≤ 2% [23].

Limits of quantitation (LOQ) and Limits of detection (LOD): LOD and LOQ are expressed as the analyte concentration in the sample, typically in parts per million. The LOD was determined using the signal-to-noise (S/N) ratio. The LOQ was defined as the minimum analyte concentration that could be accurately and precisely detected and quantified under the established experimental conditions.

RESULTS AND DISCUSSION

The primary aim of the HPLC method is to isolate and quantify target analyte for contaminants and excipients. Identifying CQAs is crucial for ensuring quality of developed method.

Risk assessment

Risk Estimation Matrix (REM): The aspects posing the highest risk were identified by developing a REM and categorizing them as low, medium and high-risk factors (Table 6).

Design of Experiment: Custom design

The model fit was evaluated using data from all 08 runs (Table 7), enhancing method's robustness. In accordance with Figure 01, at a significance threshold of $p<0.01$, $R^2 = 0.72$ and $p = 0.01289$ for response retention time, $R^2 = 0.91$ and $p = 0.0160$ for response tailing factor, and $R^2 = 0.93$ and $p = 0.0084$ for response plate count, which were found to be statistically significant. The highest R^2 values and low p values suggest that the model being evaluated is a good fit for the data (Figure 01).

Statistical optimization of selected responses of the HPLC method

Numerical optimization: The prediction profiler reveals a constant relationship between parameters and responses, revealing variability in extreme results. Figure.02 shows a global desirability value of 78.3%, indicating the model's validity for achieving desired goals for all three responses [Table 08]. The results obtained for the final optimized chromatographic conditions is presented in the table 09 and the figure 03 represents the chromatogram for Amivantamab.

Method validation

Specificity: Specificity was established by examining the blank chromatogram and verifying the absence of any interfering signals at the retention time of Amivantamab. No interfering peaks were observed, indicating that the technique shows a high level of specificity.

System suitability: System suitability parameters will comply with ICH guidelines requirements. The number of

USP plate count was found to be 4319, USP tailing factor 1.40 was obtained. % RSD was found to be 0.1% and the retention time of 1.982 mins was observed. To check for conditions prevailing as such as initial, for every 6 samples a bracketing standard was kept.

Accuracy: Accuracy was evaluated by performing three replicate injections each at various concentrations. The results at all three levels were within the acceptable range of 97-103%, ensuring that, the developed method meets the accuracy criteria, as presented in Table 9.

Precision: The precision of the method was evaluated to assess the consistency of data across multiple measurements. Six replicate injections at 100% concentration were performed. The % RSD value of 0.22% was well within the acceptable limit of 2%. Thus, the proposed method demonstrated a high level of precision.

Linearity: Amivantamab solutions ranging from 50 to 150 µg/mL were analyzed at 248 nm. The resulting calibration curve exhibited an R^2 value of 1.000, demonstrating excellent linearity of the method across the 50–150% concentration range for Amivantamab, as illustrated in Figure 4.

Limit of Quantification and Limit of Detection: THE LOQ and LOD were established by the S/N ratio method. The S/N values for LOQ and LOD were 3.2 and 10.7, respectively. The LOD, representing the minimum detectable concentration, was estimated to be 0.013 µg/ml, while the LOQ, indicating the lowest quantifiable concentration, was found to be 0.042 µg/ml.

Robustness: The robustness of the technique was evaluated by conducting the assay after introducing negligible deviations in composition of mobile phase composition, flow rate, temperature and pH. The results met the system suitability criteria, confirming that the established method is robust and remains unaltered by minor fluctuations in these parameters (Table 10).

CONCLUSION:

The QbD based approach enabled the systematic development and optimization of a robust RP-HPLC method for the analysis of Amivantamab in bulk form. Through a detailed risk assessment employing the Ishikawa diagram and the risk estimation matrix, the key factors affecting method performance were systematically identified and appropriately controlled. The statistical optimization by Design of Experiments (DoE) and MODR analysis resulted in chromatographic conditions that were both highly attractive and very powerful. ICH Q2(R1) guidelines were followed for the validation of the optimized method which in turn led to excellent accuracy, precision, linearity, sensitivity, robustness, and specificity. Overall, the QbD approach proved to be highly effective for the development of reliable analytical methods., and the proposed RP-HPLC method is suitable for the routine quality control of Amivantamab.

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Conflict of Interests The authors declare that there are no conflicts of interest related to this article.

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TABLES AND FIGURES

Table 1: Identification of QTPP

QTPP Element	Target	Justification
Technique	RP-HPLC	Preferred for high sensitivity, reproducibility, and wide applicability
Specificity	No interference from excipients/degradants	To ensure selective quantification of analyte
Accuracy	98–102% recovery	Ensures true value detection of analyte
Precision	%RSD ≤ 2.0%	Indicates repeatability of measurements
Linearity	$R^2 \geq 0.999$	Method should be linear over the concentration range
LOD	Low nanogram to microgram range	Detects minimum amount of analyte
LOQ	Lower than minimum quantifiable concentration	Important for impurity profiling
Run time	< 10 minutes	Reduces analysis time, improves efficiency
Robustness	Stable under small method variations	Ensures method reliability
System suitability	Meets resolution, NTP, tailing, etc.	Verifies system readiness before analysis

TABLE 2: Critical quality attributes (CQAS)

Parameters	Target	Justification
Retention time	+/-5min	As per ICH guidelines
Theoretical plate	>2500	As per ICH guidelines

Tailing factor	<2	As per ICH guidelines
Flowrate	≤1min/ml	According to ICH recommendations, the ideal flow rate for the mobile phase was discovered to be 1-1.2ml/min
Column temperature	25±2°C	The column temperature (25±2°C) as for ICH guidelines.
Peak area	Depends on concentration	The concentration of the substance that a peak represents is measured by the area under the peak.

TABLE 3: Critical method parameters (CMPS)

Parameters	Target	Justification
Column selection	GL Sciences C8 column (150 × 4.6 mm, 5 µm)	New method development, functional group base material.
Injection volume	10µl	The average injection volume (in1/injection) must be 2ml +0.4 l in order to meet the acceptance criteria.
Column temperature	25±2°C	The column temperature (25±2°C) as for ICH guidelines.
Flow rate	<1ml/min	According to ICH recommendations, the ideal flow rate for the mobile phase was discovered to be 1-1.2ml/min.
Buffer type	Organic solvents	Organic solvents must be added to the eluent to reduce hydrophobic adsorption on the polymeric stationary phase and increase analyte solubility.
Detector (UV)	248 nm	Where the analyte shows maximum absorbance.
Buffer Ph	2-8 pH	For reversed-phase separations using silica-based columns, the normal pH range is between 2and 8.
Buffer concentration	KH_2PO_4	For reversed-phase separations using silica-based columns, the normal pH range is between 2 and8.
Elution method	Isocratic mode	Maintain as constant mobile phase (same polarity).
Mobile phase composition	KH_2PO_4: Methanol	Mobile phase should have polarity that is compatible sample components.

CMPs	Units	Lower limit	Upper limit
Buffer concentration in mobile phase	%	50	80
Column type	-	Gl sciences 150	Inertsil 250
Flow rate	ml/min	0.5	1.0

Table 4. Design summary of custom design

TABLE 5: Final optimized RP-HPLC chromatographic condition

Sl No	Parameters

Mobile Phase Composition	KH_2PO_4 (80%): Methanol (20%)
Flow rate	1ml/min
Column type	Gl science C8

TABLE 6: RISK ESTIMATION MATRIX (REM)

CMAS / CQAs	Retention time	Tailing factor	Plate count	Resolution	Sensitivity
Flow rate	High	Medium	Medium	Medium	Medium
Run time	Medium	Low	Low	Medium	Medium
Mobile phase composition	High	High	High	High	Medium
Temperature	Medium	Low	Low	Low	Low
Column type	High	High	High	Medium	High
Wavelength	Low	Low	Low	Low	High
Injection Volume	Low	Low	Low	Low	High
Isocratic elution	Low	Medium	Medium	Medium	Low

TABLE 7: METHOD RUNS AS PER CUSTOM DESIGN

Sl.no	Factors			Responses		
	Column type	Buffer concentration in mobile phase	Flow rate	Retention time	Tailing factor	USP Plate count
1	Inertsil C_{18}	80	1	1.8	1.5	2544
2	Gl sciences C_8	50	0.5	3.0	1.2	7350
3	Gl sciences C_8	80	0.5	3.3	1.3	7500
4	Gl sciences C_8	50	1	2.5	1.2	5934
5	Inertsil C_{18}	50	0.5	4.9	1.2	5398
6	Gl sciences C_8	80	1	2.5	1.2	5124
7	Inertsil C_{18}	80	0.5	4.5	1.3	4238
8	Inertsil C_{18}	50	1	2.0	1.0	4465

TABLE 8: RESULTS FOR OPTIMIZED METHOD

S.no	Sample Name	RT	Area (µV*sec)	USP Plate Count	USP Tailing	S/N
01	AMIVANTAMAB	1.979	2566417	4226	1.51	1963.3

TABLE 9: Accuracy

Amivantamab Spiked level	Sample Weight	Sample Area	µg/ml added	µg/ml found	% Recovery	Mean	Accuracy
50%	62.00	1273297	4.054	4.04	100	99	Mean= 99
50%	62.00	1267116	4.054	4.02	99		
50%	62.00	1266305	4.054	4.02	99		
100%	124.00	2555122	8.108	8.11	100	100	SD=0.45
100%	124.00	2551480	8.108	8.09	100		
100%	124.00	2552796	8.108	8.10	100		
150%	186.00	3849204	12.162	12.21	100	100	%RSD= 0.4
150%	186.00	3841736	12.162	12.19	100		
150%	186.00	3845711	12.162	12.20	100		

TABLE 10: Results for the analysis of robustness

Factors	Retention time (min)	Tailing factor	Peak area
A, Flow rate (ml/min)			
0.8	1.625	1.60	2103139
1.0	2.535	1.57	3289314
Mean	2.08	1.58	2696226
B. Temperature			
25^0C	1.788	1.63	2282046
26^0C	2.230	1.59	3643757
Mean	2.0	1.61	2962901
C. pH of buffer			
4.6	1.984	1.60	2580309
4.7	1.986	1.50	2576538
Mean	1.98	1.55	2578423
D. Composition			
70:30	1.625	1.60	2103139
80:20	2.230	1.59	3643757
Mean	1.92	1.59	2873448

Figure 1: Actual vs predicted plot for selected responses

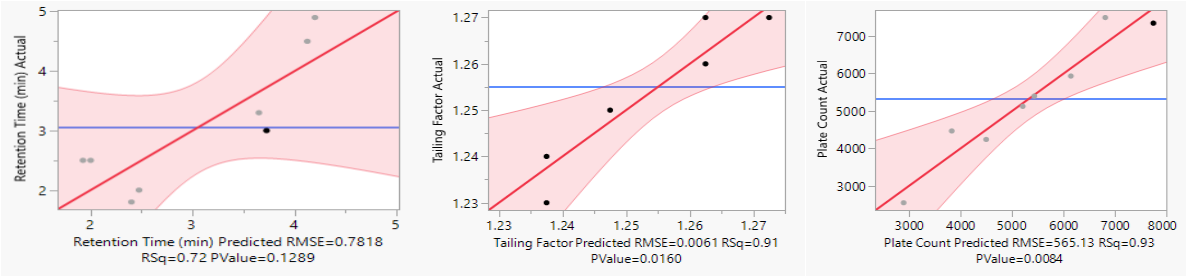


Figure 2: prediction profiler with maximized desirability

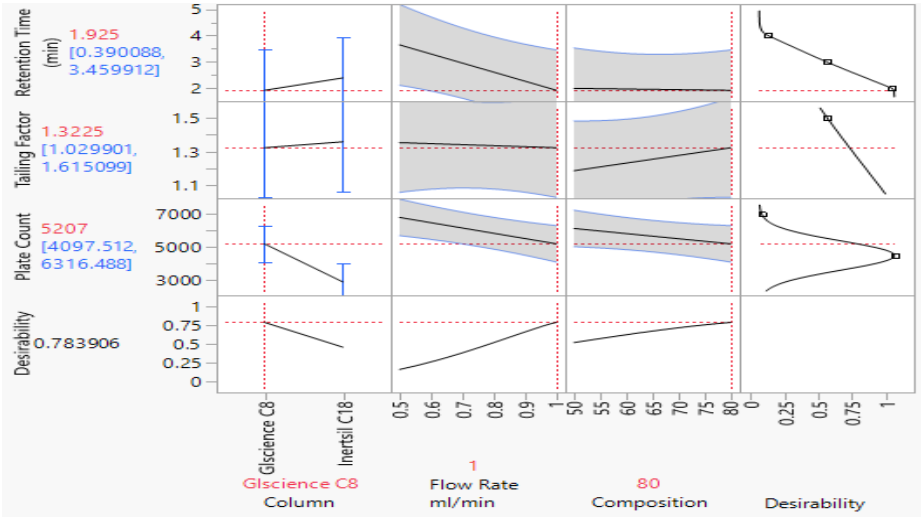


Figure 3: chromatography of the optimized method (standard chromatography)

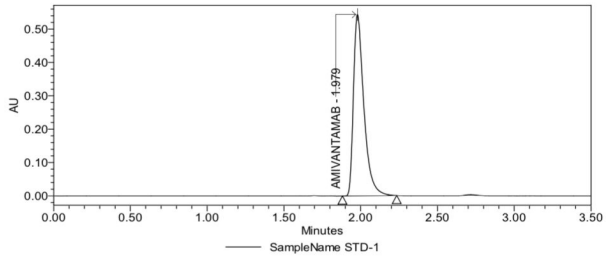


Figure 4: Linearity curve of Amivantamab by RP-HPLC

