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DEVELOPMENT AND VALIDATION OF LC/MS-MS METHOD FOR ESTIMATION OF AXITINIB FORMULATIONS BY BOX BEHNKEN DESIGN

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Abstract

Several analytical chromatographic methods have published about Axitinib drug candidate, but they are cumbersome, not easy to replicate and time tedious. In this regard, there is a need to develop QbD approach of a sensitive and rapid LC-MS/MS for the estimation of Axitinib in its formulation and validation. The separation of Axitinib was achieved using the mobile phase 10mM ammonium formate and acetonitrile in the ratio of 30:70 v/v at the flow rate of 0.87 mL/min using Zorbax C18 (50 mm x 4.6 mm i.d., 5µm) column. The critical method parameters were identified and was optimized using box behnken design. The obtained model was found to be statistically significant with a probability (p) value of less than 0.05 and composite desirability of 0.781. The method performance was evaluated as per ICH guidelines with linearity ranging from 1 ng/mL to 65 ng/mL with a correlation coefficient of 0.857. The LOD and LOQ limits were found to be 300 ng/mL and 1 ng/mL, respectively. The mean recovery was in the range of 96.66 to 100.1 %. During method transfer, the method was validated and verified for targeted method performances, robustness, and system suitability.

Keywords: Axitinib, Box behnken design, Quality by design, Liquid Chromatography-Mass Spectrometry/Mass Spectrometry, Validation.

1. INTRODUCTION

Axitinib (AG-013736)(AXB) is a potent orally active tyrosine kinase inhibitor that inhibits angiogenesis (TKI). The compound has also been shown to inhibit angiogenesis, vascular permeability, and blood flow in vitro[1,2]. In Phase III clinical trials, AXB demonstrated antitumor activity against Kidney Neoplasms[3], including renal cell carcinoma (RCC) [4,5] pancreatic cancer [6] and thyroid cancer [7]. The assay used in the first pharmacokinetic study was very brief and combined liquid chromatography–tandemmass spectrometry (LC/MS/MS) with (laborious) liquid–liquid extraction [8]. We need LC MS for this application because the separation as well as the identification can be performed simultaneously. Very fewer quantity of sample is required so economic . Highly accurate an précised , eliminates batch failure , reduces the sample cost , shorter run times , multiple compounds on a single run can be analysed. Ángeles et al., have demonstrated the oxidized lipids in the metabolic profiling of neuroendocrine tumors by utilizing RP-LC-ESI-QTOF-MS/MS [9]. Huynh HHet al., have developed and validated the simultaneous quantification method of 14 tyrosine kinase inhibitors in human plasma using LC-MS/MS [10]. Yu He et al., have developed and validated the eight tyrosine kinase inhibitors by utilizing

LC-MS/MS method simultaneously with pharmacokinetic studies [11]. The higher sensitivity of this approach compared to other TKI drugs may be due to the use of a new generation LC system and column with higher pressures and sub-2 m particles (Ultra Performance LC), which had not previously been used for TKI drugs with QbD approach.

The pharmaceutical industry has prioritized product efficiency, safety, and efficacy as the most important requirements for developing new policies that can supplement or replace current quality and risk management systems[12]. For any entity, quality being the basic criteria is given importance by all regulatory bodies .The development of a new drug product consists of many pharmaceutical process, including analytical testing [13]. The analytical data generated support further decision on how development should be pursued [14]. Analytical method failure is becoming more frequent these days, especially during method transition. In spite of analytical specifications, interferences might occur from the lab environment, analyte, analyst or instrument [15]. Robustness and ruggedness should be developed early in the system creation process to ensure that the system performs well over the product's lifetime[16]. If not introduced early enough, it could be appropriate to redevelop, revalidate, and retransfer methodological processes, which would take time and money [17]. In certain nations, QbD has been made obligatory, which ensures that product and process efficiency characteristics must be technically engineered to achieve particular targets [18]. The analytical quality by design outlines the activities that should be performed early in a analytical development before initiating validation [19]. A few LC-MS/MS analytical methods for the drug Axb were published, and nowhere box behnken design QbD methodology was documented on the advancement of analytical methods for the quantification of Axb in formulations using LC-MS/MS [20-25].

The design of experiments (DoE) is an important tool in the implementation of systematic chromatographic methods in QbD. It not only assists in the identification of method variables that have a major effect on method efficiency, but it also makes it easier to refine method variables to save time, effort, and resources. Several literature studies exist in this respect, demonstrating the greater success of the QbD methodology for the efficient development of chromatographic methods with greater versatility and improved process efficiency [26-30].

The research work's aim is therefore different in terms of its implementations, since it is the first time that the QbD technique has been used in systematic process creation and optimization studies. As a result, attempts were made to develop a novel LC-MS/MS approach for estimating Axitinib in order to study a particular solvent system. Furthermore, the designed method was optimized using a carefully chosen experimental design and validated using the ICH-recommended conditions for assessing process suitability and stability. Through the QbD methodology for the analytical process production of Axb in its formulations, a new method was developed and validated that is highly responsive and cost-effective.

2. Experimental

2.1 Standards and Reagents

Pure Axitinib was obtained as a gift sample from MSN laboratories, Hyderabad, India. The commercially available tablet formulation (INLYTA 1 mg) was procured from Pfizer. Acetonitrile and Formic acid analytical grade were purchased from Sigma Aldrich and Rankem Fine Chemical Limited respectively. Analytical grade buffer salt Ammonium phosphte was used.

2.2 Instrumentation

Shimadzu LC-MS/MS 8030 system with electro spray ionization interface were used. We have utilize the LC-20AD pump, SPD-M20 PDA detector, CTO-20AC column oven, CBM-20 alite controller, SIL-20AC auto sampler and 20AC auto sampler. Lab Solutions software was used to develop the process. The chromatographic separation was performed using Zorbax C18 (50 mm x 4.6 mm i.d., 5 m) as a stationary phase in isocratic elution mode with 10 mM Ammonium formate (pH- 4.5) : acetonitrile in the ratio of 30:70 (v/v) with a flow rate of 0.87 ml/min and an injection of 30 µl whilst maintaining the column ambient temperature.

2.3 Preparation of standard stock solution

A solution of 2mg/ml of Axb was prepared by dissolving 2 mg of Axb in 2 mL of acetonitrile, labelling, and storing the solution below 8°C solution of 2 mg/mL of Axb was prepared. To prepare a 10 g/mL solution for LC-MS/MS, 0.1 mL of a 1 mg/mL Axb solution was diluted in acetonitrile to a 10 mL normal volumetric flask. Stock solution b was used to render calibration working solutions by dilution with methanol to achieve concentrations of Axb ranging from 1 ng/mL to 65 ng/mL. Samples were prepared in bulk at 3 ng/mL (LQC), 20 ng/mL (MQC), and 59 ng/mL concentrations (HQC) for quality management.

2.4 Preparation of sample solution

Five tablets were weighed precisely and powdered, and a weight of the powder equal to 10 mg of Axb was transferred to two 10 mL volumetric flasks, where the contents were dissolved with acetonitrile and purified. To achieve a concentration of 10g/mL of Axb, the filtered solutions were rendered up to volume with acetonitrile. The above solutions were further diluted with methanol to achieve a concentration of 3 ng/mL, 20 ng/mL, 59 ng/mL (LQC, MQC and HQC).

2.5 Method Development

To achieve the ATP's criteria, the Analytical Target Profile (ATP) approach and system conditions were chosen. To better understand the functional relationship between device input variables and process output characteristics, risk assessment studies were conducted.Data collected during the method's development and early use was used to inform a risk evaluation. which utilized the tools like the Fishbone diagram and the Relative Risk Matrix Analysis (RRMA) to decide which variables should be analyzed and which should be managed.

2.6 Experimental design

The QbD method optimization was carried out using the software Design expert version 7.0 (Stat-Ease). An optimization experimental Box behnken design was opted for the study. Based on the preliminary studies five factors were chosen as the critical parameters (pH of mobile phase, volume of injection, % of organic phase, flow rate and heat block temperature) having a strong effect on the responses like peak area, tailing factor, retention time. DoE was utilized to fit the output data to the required responses, and the factors were chosen as the variables to be evaluated in 42 experimental runs.

2.7 Analytical Method Validation

The ICH Q2 guidelines were utilized to measure specificity, selectivity, linearity, and range, as well as accuracy, precisions, robustness, detection limit, and quantification limit. To ensure its suitability for its intended purpose, the procedure was validated in compliance with the ICH guideline Q2 (R1) for system suitability, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), percentage recovery, and robustness.

2.7.1 Linearity

A linear model describes the relationship between a continuous response variable and the explanatory variables using a linear function. The evolved method's linearity refers to its ability to produce test results that are equal to analyte concentrations in samples within a given range. Pipetting out 0.02, 0.2, 0.5, 0.8, 1.1, 1.3 ml stock solution in 2 ml and amount making up to the mark was used to make the serial dilution of the medication. For 10 minutes, the resulting solutions were sonicated. Finally, both of these solutions were injected into pre-determined chromatographic settings, and the region equal to each concentration was calculated. To validate the linearity, a calibration curve was built between concentrations and peak area.

2.7.2 Precision

Three different amounts of Axb (LQC: 3 ng/mL, MQC: 20 ng/mL, and HQC: 59 ng/mL) were measured at different times on the same day to determine precision (intra-day). followed by a second day of repetition (i.e., inter-day or intermediate precision).

2.7.3 Accuracy

Three QC standards, 3, 20, and 59 g/ml, were chosen from the calibration range. SD, % RSD, and Standard error of mean were also measured to ensure that data are correct within the specified range.

2.7.4 LOD and LOQ

The lowest quantity of analyte in a sample that can be detected but not generally quantified as

an exact value is the detection limit of an individual analytical technique. The LOD is defined as a concentration at a given signal-to-noise ratio.The lowest amount of analyte in a sample that can be quantitatively measured with sufficient precision and accuracy is the quantification limit of an individual analysis methods [20].

2.7.5 Robustness

The robustness of an analytical system is an indicator of its ability to remain unchanged by minor yet deliberate changes in method parameters during regular use.

3. Results

3.1 Preliminary studies

The preliminary tests for designing the LC-MS/MS system for estimating Axb were carried out according to the literature reports. The mass spectra obtained has been shown in Fig1. Initially, acetonitrile, methanol, and buffer species containing ammonium acetate and ammonium formate (each at 20 and 50 mM strength) with differing pH (between 3.0 and 5.0) and variable flow rate (between 0 and 100 mL/min) were used to measure different combinations of mobile phase 0.5 and 2.0 mL/min). Because of the quick chromatographic separation (i.e., lower Rt) seen in Fig 2, preliminary studies proposed using acetonitrile and formate buffer (pH 3.0) as an appropriate mobile phase mixture.

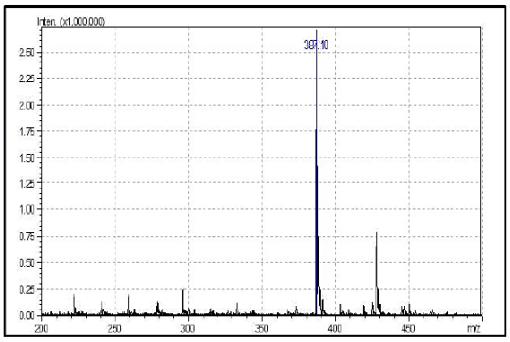

Figure 1. Mass scan spectra of the axitinib in positive mode

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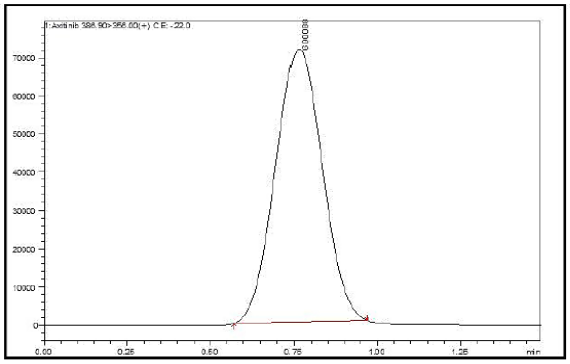

Figure 2. MRM standard chromatogram of axitinib

56x40mm (300 x 300 DPI)

3.2 Factor Screening Studies

ATP describes the method requirements which are expected to be measured.

In other words ATP states method's purpose which further initiates method selection, design and development activities. To construct the ATP, it is necessary to identify the characteristics of method that will be indicators of method performance. Once these characteristics are identified the next stage is to determine the acceptance criteria of the characteristics.

The ATP is defined with the help of knowledge and scientific understanding of the analytical process. The objectives of the method specifications is to recognise the critical factors that are likely to affect the method performance. Factor screening experiments were performed using Critical Method Parameters (CMP) and Taguchi architecture on the factors short-listed from the Risk Estimation Matrix (REM) studies. Based on the literature review we have coded the high and low values for CMVs. The first-order polynomial model was used to calculate the major effects as well as the interaction effect. The effect of variables such as mobile phase ratio, buffer pH, and oven temperature on the CAAs shown in Table I was statistically important (P 0.05) according to Pareto ranking analysis.

Table I : *Design Matrix as per BBD optimization of Axitinib*

Study type Initial design Design model	Response surface Box-Behnken Quadratic				
Factor	Name	Units	Type	Low coded	High coded
A	Buffer pH		Numeric	2.50	4.50
B	Acetonitrile	%	Numeric	70	90
C	Flow rate	ml/min	Numeric	0.50	1
D	Heat Block Temperature	C	Numeric	200	400
E	Injection volume	µl	Numeric	10	30

The method variables which are likely to affect the method performance are elution type, noise, solution stability, cone voltage, resolution along with what have been selected for this current study. There are factors which can be self optimized by the instruments so only the factors which are having a high risk on the method performance were taken into consideration.

AQbD approach involves the risk identification at early stages of development followed by appropriate mitigation plans with control strategies that will be established. In general, Ishikawa fishbone diagram can be used for risk identification and assessment.

Initially the factors were chosen which are likely to affect the method performance and then risk assessment studies were performed. The risk identification was carried out by Ishikawa fishbone diagram which helped us to identify the category of the factor .Initially many factors were chosen , but after the risk analysis only the factors with high risk on the method performance were considered. There are some factors which can be optimized by the instrument itself and some factors which had low risk on the method performance, so those were not taken into consideration for optimization The risk assessment was performed by using Relative Risk Matrix Analysis which first identifies the risk and then categorises the factors into high, medium and low risk based on their affect on the method performance. The factors having low an medium risks are accepted and no further investigation is needed whereas the factors with high risks are not acceptable and needs to be further investigated.

3.3 Method optimization

Axb at a concentration of 100 ng/ml was used in 42 experimental runs shown in Table II. The data was evaluated using multiple regression by comparing the real different expected plots, fit description analysis, analysis variances (ANOVA) (0.05), lack of fit (> 0.05), coefficient of co relation (R2), modified and predicted R2.The ANOVA for the Response Surface Quadratic and Linear model which was found to be significant and the Lack of fit was found to be non – significant. Further, the responses (Peak Area, Retention Time and Tailing Factor) shown in Table III were analysed by the model graphs (3 –D) shown in Fig 3, Fig 4, Fig 5. Peak Area and Retention Time were optimized using a Quadratic model, while Tailing Factor was optimized using a Linear model, according to the technique (Fit Summary).

Table II : *Box Behnken design of Axb*

Run	Factor1A: Buffer pH	Factor 2 B: Acetonitrile %	Factor 3 Flow rate ml/min	Factor4 D: Heat Block Temperature	Factor 5 E: Injection volume µl	Response 1 Peak Area	Response 2 Retention time	Response 3 Tailing factor
1	3.50	80.00	1.00	300.00	10.00	38413	0.642	1.5
2	3.50	70.00	0.75	300.00	10.00	46780	0.893	1.22
3	3.50	80.00	1.00	300.00	30.00	128838	0.653	1.25
4	3.50	90.00	0.75	200.00	20.00	243497	0.883	0.9
5	2.50	80.00	0.75	300.00	10.00	58651	0.857	1.16
6	2.50	80.00	0.50	300.00	20.00	211272	1.303	1.12
7	2.50	90.00	0.75	300.00	20.00	250299	0.869	0.9
8	4.50	80.00	1.00	300.00	20.00	196712	0.638	1.07
9	3.50	80.00	0.75	300.00	20.00	161191	0.858	1.29
10	2.50	80.00	0.75	400.00	20.00	170058	0.869	1.2
11	3.50	80.00	0.50	300.00	10.00	137883	1.27	1.3
12	3.50	80.00	0.50	300.00	30.00	446418	1.302	1.03
13	4.50	80.00	0.75	200.00	20.00	316196	0.85	1.26
14	3.50	90.00	1.00	300.00	20.00	161088	0.65	0.99
15	3.50	80.00	0.75	400.00	30.00	271535	0.862	1.53
16	3.50	80.00	0.50	200.00	20.00	333382	1.291	1.09
17	2.50	80.00	0.75	300.00	30.00	255755	0.876	1.15
18	4.50	90.00	0.75	300.00	20.00	363257	0.87	1
19	3.50	70.00	0.75	300.00	30.00	197814	0.896	1.26
20	3.50	80.00	0.75	200.00	20.00	104693	0.85	1.25
21	3.50	80.00	0.75	300.00	30.00	204820	0.86	1.23
22	3.50	70.00	1.00	300.00	10.00	83196	0.66	1.6
23	3.50	90.00	0.50	300.00	20.00	260643	1.309	1.72
24	3.50	80.00	1.00	200.00	20.00	105515	0.644	1.07
25	3.50	80.00	0.50	400.00	20.00	355090	1.285	1.52
26	3.50	70.00	0.75	400.00	20.00	167280	0.875	1.25
27	3.50	90.00	0.75	400.00	20.00	307962	0.845	1
28	2.50	80.00	0.75	200.00	20.00	156749	0.866	1.18

29	2.50	80.00	1.00	300.00	20.00	101339	0.649	1.17
30	3.50	80.00	0.75	200.00	20.00	273009	0.864	1.25
31	3.50	90.00	0.75	300.00	20.00	463892	0.862	1
32	4.50	70.00	0.75	300.00	30.00	335901	0.891	1.39
33	4.50	80.00	0.75	400.00	30.00	368578	0.854	1.36
34	3.50	80.00	0.75	400.00	20.00	75584	0.854	1.5
35	2.50	70.00	0.75	300.00	20.00	123788	0906	1.25
36	3.50	70.00	0.50	300.00	10.00	259543	1.341	1.28
37	4.50	80.00	0.75	300.00	30.00	504299	0.863	1.16
38	3.50	80.00	1.00	400.00	20.00	141231	0.643	1.12
39	3.50	70.00	0.75	200.00	20.00	187638	0.886	1.32
40	4.50	80.00	0.75	300.00	10.00	182578	0.845	1.37
41	3.50	90.00	0.75	300.00	10.00	181469	0.855	1.2
42	4.50	80.00	0.50	300.00	20.00	632736	1.27	1.4

Table III: *Statistical Analysis result of the responses*

Source	Peak Area		Retention Time		Tailing factor	
	Sum of squares	p value	Sum of squares	p value	Sum of squares	p value
Model	6.515E+011	<0.0001	1.80	<0.0001	0.43	0.0189
A-BufferpH	1.545E+011	< 0.0001	8.123E-004	0.0056	0.048	0.1903
BAcetonitrile	4.412E+010	0.0002	2.601E-003	< 0.0001	0.22	0.0077
C-Flow rate	1.765E+011	< 0.0001	1.69	< 0.0001	0.030	0.3022
D-Heat block temp.	1.167E+009	0.4699	1.381E-004	0.2173	0.084	0.0870
E-Injection Vol	1.818E+011	< 0.0001	7.426E-004	0.0076	0.047	0.1953
AB	2.458E+009	0.2976	6.400E-005	0.3962	0.98	
AC	2.658E+010	0.0021	1.210E-004	0.2470	0.98	0.1991
AD	3.817E+008	0.6781	2.500E-007	0.9573	1.800E-003	
AE	3.882E+009	0.1938	2.500E-007	0.9573	1.40	
BC	1.474E+009	0.4174	1.323E-004	0.2269		
BD	1.799E+009	0.3712	1.822E-004	0.1587		
BE	4.998E+009	0.1427	4.000E-006	0.8307		
CD	4.906E+007	0.8815	6.250E-006	0.7893		
CE	1.189E+010	0.0287	8.100E-005	0.3410		
DE	1.909E+008	0.7689	9.000E-006	0.7486		
A^2	2.432E+010	0.0030	1.897E-005	0.6422		
B^2	1.830E+009	0.3672	2.008E-003	< 0.0001		
C^2	1.370E+009	0.4341	0.059	< 0.0001		
D^2	1.166E+009	0.4700	3.537E-005	0.5266		
E^2	5.083E+007	0.8794	1.160E-006	0.9083		
Residual	4.524E+010		1.792E-003			
Lack of Fit	4.429E+010	0.4804	1.790E-003	0.1173		
Pure Error	9.517E+008		2.000E-006			
Cor Total	6.967E+011		1.80			

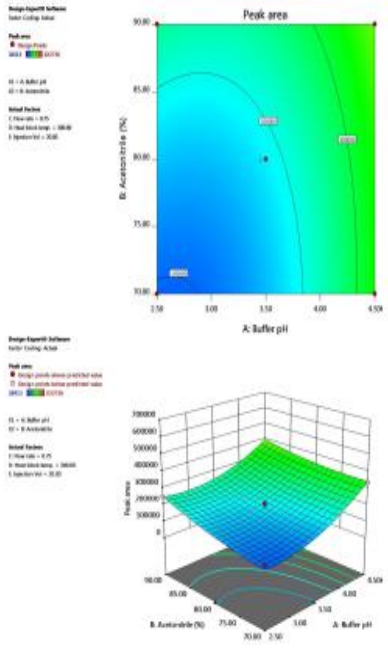

Figure 3.2D contour and 3D response surface plot showing the effect of critical method parameters on the responses

90x129mm (300 x 300 DPI)

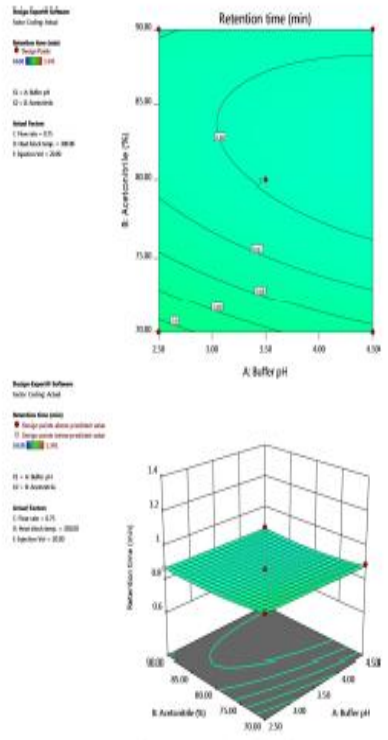

Figure 4. 2D contour and 3D response surface plot showing the effect of critical method parameters on the responses

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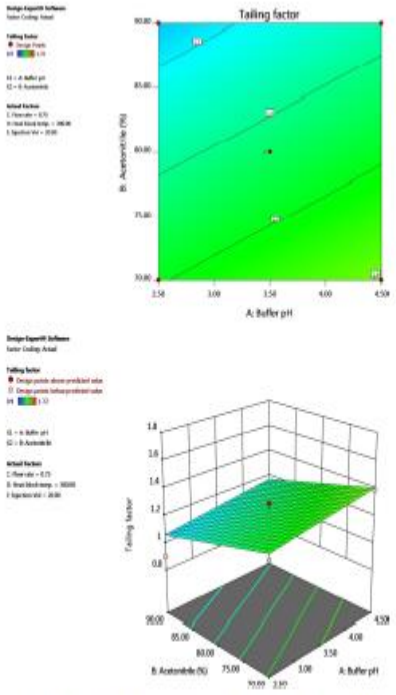

Figure 5. 2D contour and 3D response surface plot showing the effect of critical method parameters on the responses

90x129mm (300 x 300 DPI)

3.4 Optimizing data analysis

DoE version 7 was utilized to optimized the 42 runs. The response surface methodology, the optimized values of pH of mobile phase (4.5), volume of injection (30µl), % of organic phase (89.92%), flow rate (0.87 ml/min) and heat block temperature (399 C). The numerical desirability parameter, which was found to be 0.781 by the design space area shown in Fig 6, was used to identify the optimum chromatographic solution. An experimental run was carried out with the obtained optimized values with 95% confidence interval.

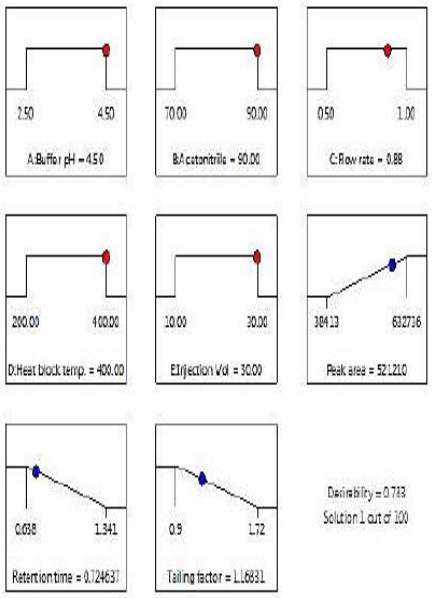

Figure 6. Numerical optimization with the highest desirability

56x40mm (300 x 300 DPI)

3.5 Response surface mapping

2D contour plots and 3D reaction surface plots for each response, such as peak area, retention time, and tailing factor. On the method CAAs, the response surface analysis showed that the analyzed CMPs had a high degree of interaction. The curvilinear response surfaces were observed portraying the effect of CMPs (i.e., ACN%, buffer pH) on peak area as the CAA (Figure 3). In this regard, an increase in the levels of the ACN % and buffer pH revealed a decrease in the values of peak area. The influence of CMPs on the CAA (i.e., Rt) was distinctly different (Figure 4). Both factors showed highly significant influence on the Rt up to intermediate levels followed by a dip. As illustrated in Figure 5, a curvilinear trend was observed for the effect of the ACN% and the buffer pH on the tailing factor with appearance of a typical “rising ridge” type trend.

MODR is a Systematic Series of Experiments, in which purposeful changes are made to input factors to identify causes for significant changes in the output responses and Determining the relationship between factors & responses to evaluate all the potential factors simultaneously, systematically and speedily

3.6 Validation

The main aim of the validation process is to put the system to the test and assess the method's allowable variability for the requirements used to operate it. The elements of validation methods and the process used to validate the system were previously discussed. The findings obtained after using the QbD method are discussed in this section. For Axb, the detection was carried out in MRM mode utilizing electro spray ionization (ESI) in positive mode.

3.6.1 Linearity

By evaluating serial dilutions of medication between 1 and 65 ng/mL and plotting the peak region against concentration, the developed method's linearity was calculated. Furthermore, by analyzing the expected and observed responses, the method's linearity was verified using least square regression analysis on the obtained results shown in Fig 7 and Table IV.

Table IV: *Linearity study of Axitinib*

Sl/No.	Concentrations of Axb (ng/ml)	Peak Area for Axb
1.	1	79977
2.	10	139987
3.	25	238769
4.	40	244676
5.	55	419057
6.	65	690088

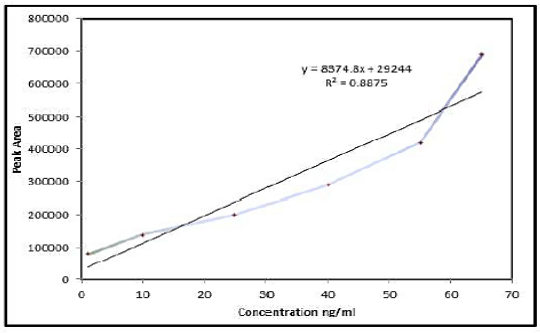

Figure 7. Calibration curve of axitinib

56x40mm (300 x 300 DPI)

3.6.2 Accuracy

In accuracy tests, low (LQC), medium (MQC), and high (HQC) quality controls were prepared. The method's accuracy was measured using the mean percentage recovery from a 100 ng/mL standard solution spiked with 80, 100, and 120 % more Axb. In addition, SD, % RSD was found to be 0.876, 0.189, 0.168 to maintain data accuracy during the study under the limits set forth shown in Table V.

Table V: *Accuracy study of Axitinib*

Standard concentration (ng/ml)	**Amount recovered ng/ml±SD**	**Recovery (%)**	**RSD (%)**
LQC:3	2.90±0.02	96.66	0.876
MQC:20	19.58±0.037	97.6	0.189
HQC:59	58.98±0.01	98.8	0.168

3.6.3 Precision

The precision of the procedure was calculated by calculating three different concentrations of Axb (LQC: 3 ng/mL, MQC: 20 ng/mL, and HQC: 59 ng/mL) at different times on the same day (intra-day precision or repeatability), then repeating the experiment the next day (i.e., inter-day or intermediate precision). The magnitudes of mean percent regeneration, SD, and % RSD was found to be 0.742, 0.691,0.627 and 0.965,0.956,0.337 for intraday and interday respectively..It was discovered that the percentage recovery ranged from 99.51 to 101.34 percent. These values have a coefficient of variation of less than 2%. This procedure was found to be reliable ,robust and effective because of Axb complete recovery shown in Table VI.

Table VI: *Precision study of Axitinib*

Standard concentration (ng/ml)	**Amount recovered ng/ml+SD**	**Recovery %**	**RSD %**
Intraday Precision LQC: 3 MQC: 20 HQC: 59	2.92 ±0.21 20.31±0.14 58.67 ±0.37	97.33 101..55 99.44	0.742 0.691 0.627
Interday Precision LQC: 3 MQC:20 HQC: 59	2.97±0.028 19.32±0.187 59.04±0.198	97 96.6 100.06	0.965 0.956 0.337

3.6.4 Assay of Tablets

Five tablets were weighed and thoroughly powdered, and a weight of powder equal to 10 mg Axb was transferred to 10 ml volumetric flask. The contents were dissolved with acetonitrile and filtered. The filtered solutions were diluted to get a Conc.10 g/ml of Axb. Further the above solutions were diluted with acetonitrile to get a conc of 3, 20, 59 ng/ml (LQC, MQC and HQC). The results are shown in Table VII.

Table VII: *Assay and Recovery study for Axitinib formulations*

Formulation	**Label claim**	**Amount taken for assay(ng/ml)**	**Amount found ±SD**	**Found mg/tab**	**Recovery %**
Axb (Inlyta)	1mg	3	2.9 ± 0.2	0.98	96.66
		20	19.58±0.45		97.9
		59	59.1±0.83		100.1

3.6.5 LOD and LOQ

The limit of detection (LOD) and limit of quantification(LOQ) was determined for Axb, which was found to be 300 ng/ml, and 1 ng/ml with signal-to-noise ratio of 3:1, and 10:1 respectively shown in Table VIII. Reagents and the use of non-LC-MS/MS grade solvents can all influence these values, resulting in changes in signal to noise ratios. As a result of this finding, the developed method has a high sensitivity.

Table VIII: *LOD and LOQ study*

S.No.	Parameters	Values obtained for Axb
1	LOD (ng/ml)	300 ng/ml
2	LOQ (ng/ml)	1 ng/ml

3.6.6 Ruggedness and Robustness

Changing the experimental conditions was used to investigate the method ruggedness and robustness. When the experimental conditions such as operators, reagent source, column of similar type, and optimized conditions were changed. But there was no significant differences in the chromatographic parameters were observed.

4. Discussion

The improved analysis ensures the developed method is robust , precise with better system suitability. Besides to determine failure more, the process with in designs spaces allows continuous improvement. Response surface analysis provides the researchers, academicians, and pharmaceutical industries to better understand the factor–response relationship and its interactions. Current research demonstrated the efficient implementation of QbD concepts for the development of an enhanced robust and efficiency LC-MS/MS system for AXb. Based on the initial screening trials, the highly critical factors were identified and then tailored for ameliorate the method robustness. In extreme variation the key variables that affect process performance, extensive validation studies have ensured a high degree of system robustness. Moreover, the system's sensitivity to the AXb was much greater than the r values.

5. Conclusion

During a method development robustness and ruggedness should be established early to make certain method performances over the lifetime of the product. Inspite of the analytical specifications interferences might occur. The QbD approach delivers the formation of a design space which ensures the recognition of critical factors at the early stage of development before the validation initiates. The current research work ensured reduction in variability in analytical attributes and has improved method robustness and higher efficiency. The detailed QbD method for evaluating Axb and its purpose in risk evaluation studies aided in prior the critical factors influences the process parameters, resulting in a stable-indicating analytical tool that is accurate, fast, rapid, responsive, and cost-effective. Eventually, factors selection and optimization studies using laboratory designs lead to the selection of CMPs.For estimating Axb commercial formulations of conc. varying from 1 ng/mL to 65 ng/mL, the existing method was developed and validated. The data obtained was found to be more robust and well within the specified parameters. The results of the device suitability studies showed that this method is suitable for studying drugs in formulations.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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