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DEVELOPMENT AND VALIDATION OF REVERSE PHASE HPLC METHOD FOR ALECTINIB WITH QBD APPROACH AND IT'S FORCED DEGRADATION STUDIES

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ABSTRACT

One of the most common types of cancer is lung cancer, which frequently manifests as locally progressed or metastatic illness at the time of diagnosis. Adults with metastatic Non-small Cell lung cancer (NSCLC) that is ALK-positive should take ALECINIB. In order to assess Alectinib, we created and verified an isocratic high-performance liquid chromatography method in this work. Drug quantitation and selectivity are possible using this approach. A passive Cosmoil C18 analytical column (4.6 X 150 mm, 5 µm) was used to validate the procedure. The equilibration phase included methanol and water (80:20% v/v) at a pH ratio of 3. The temperature of the column was kept at room temperature, and the flow rate was 1 mL/min. The wavelength of the photodiode array detector was adjusted to 267 nm. In the concentration range of 10, 20, 30, 40, and 50 ppm, the calibration curve is linear, and the correlation coefficient (r^2) is 0.999. The percentage RSD for intra- and inter-day precision was 0.189 and 0.106%, respectively. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.0747 µg/mL and 0.2264 µg/mL, respectively. The precision, accuracy, linearity, precision, limit of detection, quantification, and technique strength are all validated for this approach. This method can be utilized for Alectinib analysis and estimate in active pharmaceutical and medications because of its accuracy and speed. Stress degradation for Alectinib was carried out and it showed degradation in every condition. It showed higher degradation in base hydrolysis with upto 23.64 % degradation and upto 17.04% degradation in Acidic condition. In the oxidation condition, heat degradation and Photolytic conditions the degradation was found to be 8.79%, 1.08% and 0.14% respectively.

KEYWORDS: Lung Cancer, Alectinib, RP-HPLC, Method development, Validation, etc.

INTRODUCTION:

When lung cancer is found to be progressed or metastatic, it is the most common cause of cancer-related mortality globally. 85% of lung cancer cases are non-small cell lung cancer (NSCLC), which is typically progressed at the time of diagnosis (1). One of the receptor tyrosine kinases in the insulin receptor family is anaplastic lymphoma kinase (ALK) (2). Numerous malignancies in humans have been linked to genetic alterations in ALK. Oncogene enhancers can be expressed when ALK is triggered by a mutation, gene amplification, or chromosomal layout (3).

Certain medical problems, such as cancer-related dysphagia or respiratory failure, meningitis, or metastases to the central lymph nodes, may make acute TKI impractical. Consequently, TKI administered intranasally might be the only source (4). In patients with advanced ALK-positive NSCLC and in patients with older ALK-positive NSCLC treated with Alectinib, it is also a highly powerful ALK inhibitor that is advised as first-line therapy. In the US, AT is authorized for the management of ALK-positive metastatic non-small cell lung cancer. Adults with metastatic NSCLC that is ALK-positive may be treated with AT in the US. In both the US and the EU, a dose of 600 mg taken twice daily is advised. In a 19-month analysis of patients who had previously received Alectinib and double platinum chemotherapy, AT was found to be superior to both chemotherapy and Alectinib as first-line therapy in a phase III trial including patients with ALK-positive NSCLC. a noteworthy increase in progression-free survival (16).

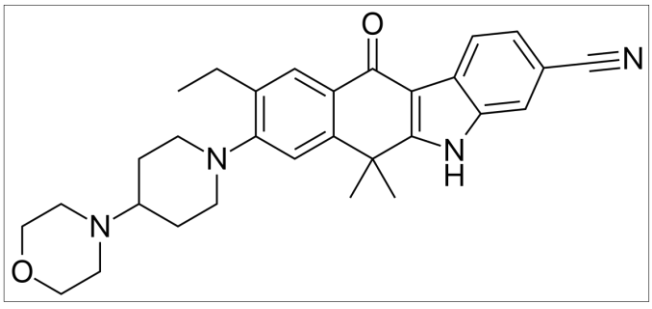


Figure 1: Molecular Structure of Alectinib

According to the literature, there are many publications on UV-Visible spectroscopy and HPLC, but none of them use quality by design. According to the ICH Q8 (R2) guidelines, the quality by

design (QbD) method was used to assess the quality of Alectinib in dosage form (1-3). RP-HPLC method should be developed for the measurement (5-6).

Since Alectinib is a new and recently synthesized drug, resources are scarce. This is because the current literature lacks the means to analyze the drug Alectinib in pharmaceutical products. In order to estimate and analyze Alectinib in pharmaceuticals, this study will create and optimize RP-HPLC settings. It will also validate the method and apply it to the routine analysis of medications and pharmaceuticals.

MATERIAL AND METHODS

Chemicals and reagents

Table 1: List of drugs used and name of its supplier

1.	Alectinib	Alecen
2.	Ortho-Phosphoric acid	Thermofisher scientific, India
3.	Methanol	Merck Specialities Pvt. Ltd., Mumbai
4.	Water	Merck Specialities Pvt. Ltd., Mumbai

INSTRUMENTATION:

Table 2: List of Instruments used

1.	HPLC	HPLC Binary Gradient System
	Software	HPLC Workstation
	Model Number	HPLC 3000 Series
	Company	Analytical Technologies Ltd.
	Detector	UV-3000-M
	Pump	P-3000-M Reciprocating (40 Mpa)
2.	Column	Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron)

3.	Analytical Balance	Wenser High Precision Balance
	Model	PGB 100
4.	Vortex machine	Remi CM 101 plus
5.	Nylon 6,6 membrane 0. 45µm 47mm Filters	Pall pvt. Ltd
6.	UV-Vis Spectrophotometer	Analytical Technologies Ltd.
	Model	2012
	Wavelength Variability	0.1 nm
	Type	Double beam
	Software	UV-VIS Analyst
	Scanning range	190 nm-1100 nm
7.	All Glass Filter Holder- 47mm (1L flask, 300ml funnel)	Borosil Glass works Ltd., Mumbai
8.	Melting Point Apparatus	Veego
9.	RC membrane 0.45µm 15mm Syringe Filters	AxivaSichem Biotech
10.	Ultra Sonicator/ water bath	Wensen Ultra Sonicator
	Model	WUS-4L
	Capacity	4 liter
11.	FTIR	Bruker FT-IR ALPHA II

METHODS

1 Preliminary Analysis of Drug: Alectinib

a) Description: The sample of Alectinib was observed for its color and texture.

b) Solubility: The sample of Alectinib was taken in test tubes and observed for solubility in water, acetonitrile, and methanol.

c) Melting Point: The sample of Alectinib was taken in capillary tube and kept in melting point apparatus.

d) FTIR: FT-IR was performed by using Bruker FT-IR ALPHA II instrument to identify the obtained Alectinib. The Alectinib sample was mixed with KBr and crushed using mortar and pestle. The mixture was then analyzed by using the instrument and graph was obtained.

HPLC Method Development

1. **Chromatographic Conditions:**
   - **a.** Oven Temp: 30℃
   - **b.** Flow rate: 0.8 ml/min.
   - **c.** Mobile Phase: Methanol: Water (80:20) pH adjusted to 3.0 using OPA
   - **d.** Runtime: 8.48 minutes
   - **e.** Injection Volume: 20µl
   - **f.** Wavelength:267 nm
   - **g.** Diluent/solvent: Methanol: Water (80:20)
   - **h.** Column: Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron)
   - **i.** Pressure:12-13MPa

2. **Standard Preparation:**
   a. Alectinib Stock Solution-I (RSS-I):

   Prepare a Alectinib Stock Solution (RSS-I) by adding 10 mg of Alectinib pure drug in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. of Alectinib = 1000 ppm).

   b. Various concentration from stock solutions were prepared as shown in table given below:

Table 3: Sample preparation for different concentration

Sr. No.	Drug	Concentration (ppm)	Volume of stock solution taken (ml.)	Final Solution Volume (ml)
1	**Alectinib**	**10**	0.1	10

2	**Alectinib**	**20**	0.2	10
3	**Alectinib**	**30**	0.3	10
4	**Alectinib**	**40**	0.4	10
5	**Alectinib**	**50**	0.5	10

3. Preparation of drug Product:

Sample Stock Solution:

i. 10 tablets were weighed and average weight was calculated. And tablets were crushed & mixed in mortar and pestle.
ii. Powder weight equivalent to 10 mg Alectinib was weighed into 10 ml volumetric flask & add 5 ml diluent, sonicate for 5 minutes and make the volume to 10 ml with diluent. (Conc. of Alectinib =1000 ppm).

4. Selection of Wavelength:

The sample was scanned from 200-400 nm with PDA detector. The Wavelength selected for analysis chosen was 267 nm on basis of appropriate intensity of Alectinib.

Development and Optimization Method using A QbD Design:

1. **Analytical Target Profile (ATP)**
2. **Critical Analytical Attributes (CAA)**
3. **Critical Method Parameters (CMP)**
    a) **Flow rate**
    b) **Injection Volume**
4. **Critical Method Material Attributes (CMMA)**

Method Validation:

a. Assay:

i. Individual injections of Alectinib API and formulation were prepared of 30 ppm and peaks were identified from Retention Time.

ii. Blank was injected to ensure there is no blank peak interfering with the main analyte peaks.

b. System Suitability:

i. System suitability parameters are as below:

- **Resolution:** Resolution value should be greater than 1.75. This parameter is applicable only when there is combination of two samples. In case of single sample, it will show '0' value.
- **Theoretical Plates:** Number of theoretical Plates should be greater than 2000. It indicates efficiency of column.
- **Tailing/ Asymmetry factor:** Value of asymmetry factor should be less than 2.

c. Accuracy:

Table 4: Recovery studies sample preparation

Sr. No.	Recovery	Conc. of formulation (ppm)	Conc. Of Std. (ppm)	Combined concentration (ppm)
1.	50% Recovery	20	10	30
2.	100% Recovery	20	20	40
3.	150% Recovery	20	30	50

d. Linearity:

Table 5: Linearity sample preparation

Concentration (ppm)	X ml of ASS-I	Diluted to
10	0.1	10 ml
20	0.2	10 ml
30	03	10 ml
40	0.4	10 ml

50	0.5	10 ml

e. LOD/ LOQ:

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$$QL = \frac{10\sigma}{S}$$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

f. Robustness:

Table 6: Robustness Change in Wavelength

Condition	Increased	Normal	Decreased
Wavelength	269 nm	267 nm	265 nm
pH	3.2	3.0	2.8

g. Inter-day Precision: Sample solutions containing 10 mg of Alectinib were analyzed at 30 ppm concentration. Alectinib different days and % RSD was calculated. It is usually expressed as standard deviation or relative standard deviation.

h. Intraday Precision: Sample solutions containing 10 mg of Alectinib at 30 ppm concentration. Alectinib were analyzed three times on the same day and %RSD was calculated.

i. Ruggedness: The pH of mobile phase was changed in (±0.2**)** proportion and the change in detection wavelength (±2 nm) **(Table 30)** and the effect of the results were examined using 20 ppm solution of Alectinib in triplicate.

j. Forced Degradation: To determine the performance of Alectinib in stressed conditions, solution of Alectinib faced to various conditions like Acid hydrolysis, base hydrolysis, oxidation, photolysis and heat degradation **(Table 32).**

RESULTS AND DISCUSSION:

Preliminary studies on Alectinib

Physical characteristics: The Alectinib API was observed visually and it is white to slight yellow colour powder.

Melting point: The procured reference standard of Alectinib was found to melt in the range of 274^0C- 276^0C.

Solubility

The drug was found to be

- Soluble in DMSO
- Poorly Soluble in water.

UV Spectroscopy

An ultraviolet spectrophotometer was used to scan the material from 190 to 1100 nanometers. For the purpose of accurately identifying Alectinib, the measurement wavelength that was used for the analysis was 276 nm.

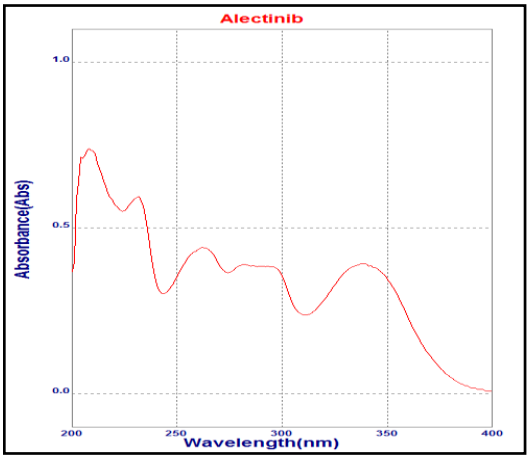


Figure 2: UV Spectrum of Alectinib

Studies on the chromatographic behaviour of Alectinib

After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity & accuracy. The optimized parameters for selected method are as below:

Table 7: Different Trials of Chromatographic Condition

Sr. No.	Column used	Mobile phase, Flow Rate and Wavelength	Inj. Vol.	Observation	Conclusion
1	Cromosil C18 (250 ×4.6mm, 5µ)	Methanol+ water (90+10 % v/v) Flow Rate 0.9 ml. 267 m	20µl	Sharp Peaks were not obtained. (peak Splitting)	Hence rejected
2	Cromosil C18 (250 ×4.6mm, 5µ)	Methanol+ water (80+20 % v/v) Flow Rate 0.8 ml. 267 m	20µl	Sharp Peaks were not obtained. (peak Splitting)	Hence rejected
For Standard					
1	Cromosil C18 (250 ×4.6mm, 5µ)	Methanol+ water (80+20 % v/v) Flow Rate 0.8 ml. 267 m	20µl	Sharp Peaks were not obtained. (peak Splitting)	Hence rejected
2	Cromosil C18 (250 ×4.6mm, 5µ)	Methanol+ water (80+20 % v/v) Flow Rate 0.8 ml. 267 m	20 µl	Sharp Peaks were not obtained (peak Splitting)	Hence rejected
3	Cromosil C18 (250 ×4.6mm, 5µ)	Methanol+ water (80+20 % v/v) Flow Rate 0.8 ml. 267 m	20 µl	Sharp Peaks were obtained	Hence Selected

Thus, from the above, it has been observed that, using mobile phase of Methanol+ water (80:20 % v/v), pH 3, 267 nm, Flow rate 0.8 ml gave adequate retention at 4.699 min with good peak shape (Theoretical plates Alectinib is 8419).

Chromatogram for Trial

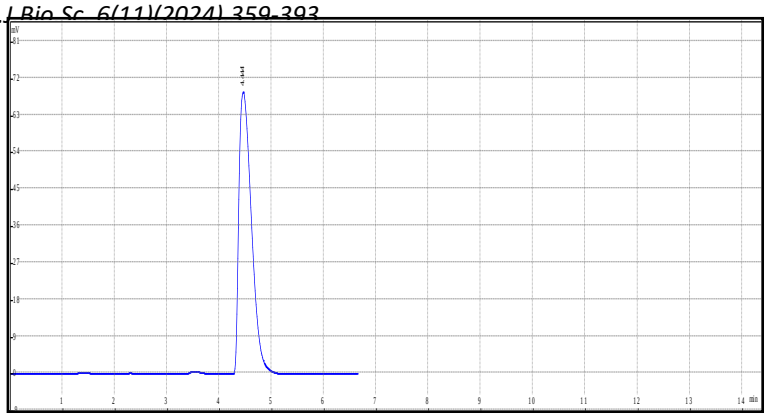


Figure 3: Chromatogram for Method development Std Alcetinib Trial 1

Table 8: Result for Chromatogram of Std Alcetinib Trial 1

Peak	Ret. Time	Area	Resolution	Theoretical Plates	Tailing Factor
1	3.292	1573103	0.000	5441	1.56

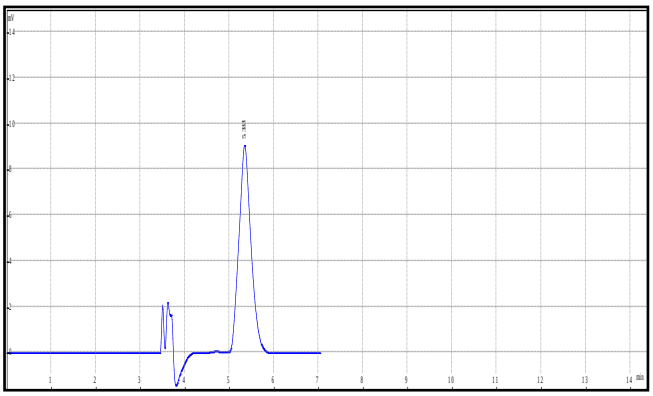


Figure 4: Chromatogram for Method development Run Trial 1

Table 9: Result for Chromatogram of Run Trial 1

Peak	Ret. Time	Area	Resolution	Theoretical Plates	Tailing Factor
1	4.444	1151535	0.000	1648	1.79

For Standard

Chromatogram for standard - Trial 1

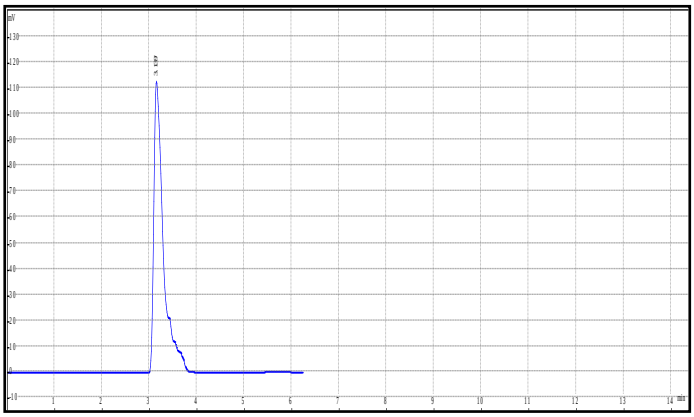


Figure 5: Chromatogram for Method development Trial

Table 10: Result for Chromatogram of Trial 1

Peak	Ret. Time	Area	Resolution	Theoretical Plates	Tailing Factor
1	3.292	1573103	0.000	5441	1.56

Chromatogram for standard - Trial 2

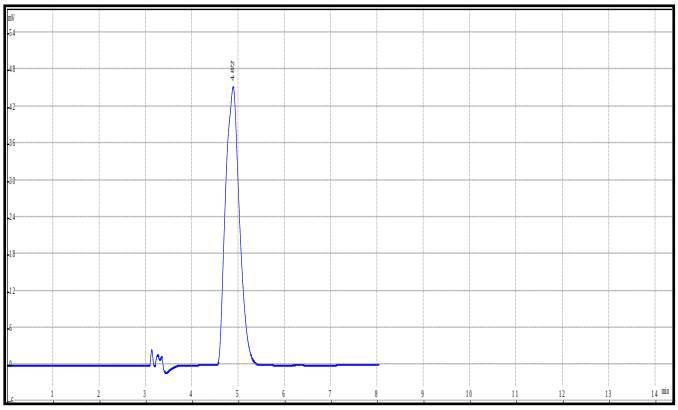


Figure 6: Chromatogram for Method development Trial 2

Table 11: Result for Chromatogram of Trial 2

Peak	Ret. Time	Area	Resolution	Theoretical Plates	Tailing Factor
1	3.139	1586130	0.000	4168	1.58

Chromatogram for standard - Trial 3

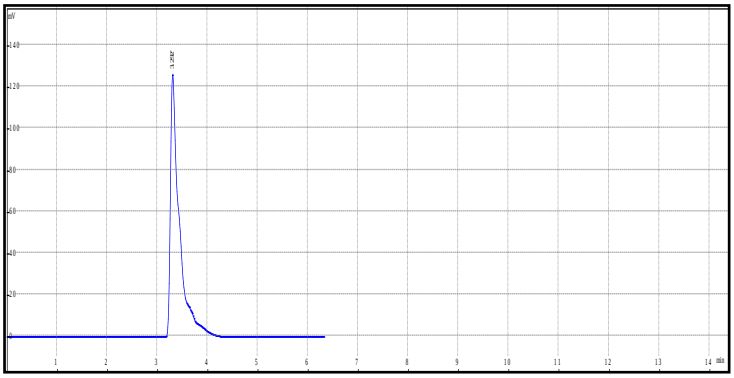


Figure 7: Chromatogram for Method development Trial 3

Table 12: Result for Chromatogram of Trial 3

Peak	Ret. Time	Area	Resolution	Theoretical Plates	Tailing Factor
1	4.872	961735	0.000	7248	1.11

9.2 Development and optimization of Method A QbD:

After initial development, a QBD analysis was done on Design expert software version 13, using Composition, Flow rate and wavelength as the variables with their upper and lower limits and in responses were retention time, peak area, Theoretical plates and peak asymmetry. Following details were shows the design expert software upates:

ANOVA response for Retention time:

Table No 13: ANOVA RESPONSE TIME

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	13.7430726	9	1.52700807	580.004964	3.26E-09
A-Composition	10.6883761	1	10.6883761	4059.77633	6.16E-11
B-Flowrate	2.230272	1	2.230272	847.126389	1.45E-08
C-Wavelength	6.13E-06	1	6.13E-06	0.00232646	0.96287753
AB	0.007744	1	0.007744	2.94141107	0.13004981
AC	0.00013225	1	0.00013225	0.05023265	0.82906011
BC	0.000121	1	0.000121	0.04595955	0.83636065
A ≤	0.73436059	1	0.73436059	278.9329	6.75E-07
B ≤	0.0565348	1	0.0565348	21.4736692	0.00238649

C ≤	0.0106848	1	0.0106848	4.058419	0.08380118
Residual	0.01842925	7	0.00263275		
Lack of Fit	0.01842925	3	0.00614308		
Pure Error	0	4	0		
Cor Total	13.7615019	16			

Fit Statistics:

Table No 14: Statistical Data

Std. Dev.	0.05131033	R≤	0.99866081
Mean	5.01035294	Adjusted R≤	0.996939
C.V. %	1.02408615	Predicted R≤	0.97857298
		Adeq Precision	85.5774802

ANOVA Quadratic Model response for Peak Area:

Table No 15: ANOVA Quadratic Model Response Peak Area

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.9055E+11	9	2.1173E+10	49.6430441	1.63E-05	significant
A-Composition	3.2675E+10	1	3.2675E+10	76.6116248	5.11E-05	
B-Flowrate	1.4094E+11	1	1.4094E+11	330.451707	3.77E-07	

C-Wavelength	508438216	1	508438216	1.19211685	0.31105077	
AB	4687319296	1	4687319296	10.9901895	0.0128504	
AC	13675204	1	13675204	0.03206376	0.86296043	
BC	1537306472	1	1537306472	3.60446736	0.09941873	
A≤	925239180	1	925239180	2.16937513	0.18426854	
B≤	9497990106	1	9497990106	22.2695968	0.0021599	
C≤	14123371.3	1	14123371.3	0.03311456	0.86076064	
Residual	2985502218	7	426500317			
Lack of Fit	2985502217	3	995167406	4975837029	1.35E-19	significant
Pure Error	0.8	4	0.2			
Cor Total	1.9354E+11	16				

Fit summary for Peak Area as per ANOVA

Table No 16: Fit Summary For ANOVA

Std. Dev.	20651.8841	R≤	0.98457427
Mean	1301276.35	Adjusted R≤	0.9647412

C.V. %	1.58704829	Predicted R≤	0.75318839
		Adeq Precision	25.7481767

ANOVA Quadratic Model response for Theoretical Plates:

Table No 17: ANOVA Model Response for Theoretical Plates

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2609196.25	3	869732.083	23.2975385	1.66E-05	significant
A-Composition	2403528.13	1	2403528.13	64.3833774	2.16E-06	
B-Flowrate	170820.125	1	170820.125	4.57576363	0.05197976	
C-Wavelength	34848	1	34848	0.93347438	0.35159258	
Residual	485309.515	13	37331.5011			
Lack of Fit	485309.515	9	53923.2794			
Pure Error	0	4	0			
Cor Total	3094505.76	16				

Fit summary for ANOVA theoretical Plates

Table No 18: Fit summary for ANOVA theoretical Plates

Std. Dev.	193.213615	R¬≤	0.84317059
Mean	7665.88235	Adjusted	0.80697919

		R¬≤	
C.V. %	2.52043544	Predicted R¬≤	0.67471657
		Adeq Precision	14.815033

ANOVA Quadratic Model response for Asymmetry: The Model F-value of 86.32 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table No 19: ANOVA Quadratic Model response for Asymmetry

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	0.0131	6	0.00218333	86.3178295	4.96E-08	significant
A-Composition	0.01125	1	0.01125	444.767442	1.28E-09	
B-Flowrate	0.0003125	1	0.0003125	12.3546512	0.0055856	
C-Wavelength	0.0010125	1	0.0010125	40.0290698	8.60E-05	
AB	0.0004	1	0.0004	15.8139535	0.00261497	
AC	1.00E-04	1	1.00E-04	3.95348837	0.07482732	
BC	2.50E-05	1	2.50E-05	0.98837209	0.34358743	
Residual	0.00025294	10	2.53E-05			

Lack of Fit	0.00025294	6	4.22E-05			
Pure Error	0	4	0			
Cor Total	0.01335294	16				

Fit summary for ANOVA Asymmetry: The Predicted R¬≤ of 0.9157 is in reasonable agreement with the Adjusted R¬≤ of 0.9697; i.e. the difference is less than 0.2. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 33.310 indicates an adequate signal. This model can be used to navigate the design space.

Table No. 20: Fit summary for ANOVA Asymmetry

Std. Dev.	0.00502933	R¬≤	0.98105727
Mean	1.31705882	Adjusted R¬≤	0.96969163
C.V. %	0.38186038	Predicted R¬≤	0.91566192
		Adeq Precision	33.309962

Points prediction form Design Expert variables and response: The Point prediction was calculated from the DOE with its expert Variables and response

Table 21: Points prediction form Design Expert variables and response

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding			
A	Composition	79.9998999	60	80	0	Actual			
B	Flowrat	0.8000	0.8	1	0	Actual			

	e	0011							
C	Wavelengh	265.00003	265	269	0	Actual			
Predicted	Predicted	CI for	Mean	99% of	Population				
Response	Mean	Median [*]	Observed	Std Dev	SE Mean	95% CI low	95% CI high	95% TI low	95% TI high
Retention Time	4.67187859	4.67187859	-	0.05131033	0.05989161	4.53025744	4.81349973	4.31123227	5.0325249
Area	1448322.57	1448322.57	-	20651.8841	24105.7604	1391321.51	1505323.64	1303166.11	1593479.03
Theoretical Plates	8426.1257	8426.1257	-	193.213615	127.259731	8151.19777	8701.05364	7461.49225	9390.75916
Asymmetry Factor	1.29205908	1.29205908	-	0.00502933	0.00547201	1.27986668	1.30425149	1.26110669	1.32301147

Based on the design expert software inputs and its output, development trials were done and the details are listed on below table:

Table 22: Design of Qbd

		Factor 1	**Factor 2**	**Factor 3**	**Response 1**	**Response 2**	**Response 3**	**Response 4**
Std	**Run**	**A:Composition**	**B:Flow rate**	**C:Wavelength**	**Retention Time**	**Area**	**Theoretical Plates**	**Asymmetry Factor**
		%	**ml/min**	**Nm**	**min**	**AU**	**Units**	**Units**
2	1	80	0.8	267	4.798	1.44E+	8631	1.29

						06		
8	2	80	0.9	269	3.972	1.20E+06	8263	1.29
6	3	80	0.9	265	3.969	1.20E+06	8352	1.27
15	4	70	0.9	267	4.783	1.29E+06	7622	1.32
10	5	70	1	265	4.365	1.21E+06	7962	1.3
4	6	80	1	267	3.571	1.08E+06	7648	1.26
7	7	60	0.9	269	6.32	1.34E+06	7094	1.37
11	8	70	0.8	269	5.343	1.42E+06	7755	1.33
13	9	70	0.9	267	4.783	1.29E+06	7622	1.32
12	10	70	1	269	4.359	1.22E+06	7455	1.32
3	11	60	1	267	5.923	1.26E+06	7028	1.36
5	12	60	0.9	265	6.34	1.35E+06	7146	1.33
17	13	70	0.9	267	4.783	1.29E+	7622	1.32

						06		
1	14	60	0.8	267	6.974	1.49E+06	7241	1.35
14	15	70	0.9	267	4.783	1.29E+06	7622	1.32
9	16	70	0.8	265	5.327	1.49E+06	7635	1.32
16	17	70	0.9	267	4.783	1.29E+06	7622	1.32

Retention Time: The Actual vs predicted graph, cube analysis and its 3D surface graph are shown below for each response as per Design expert inputs:

Retention time – Output for response:

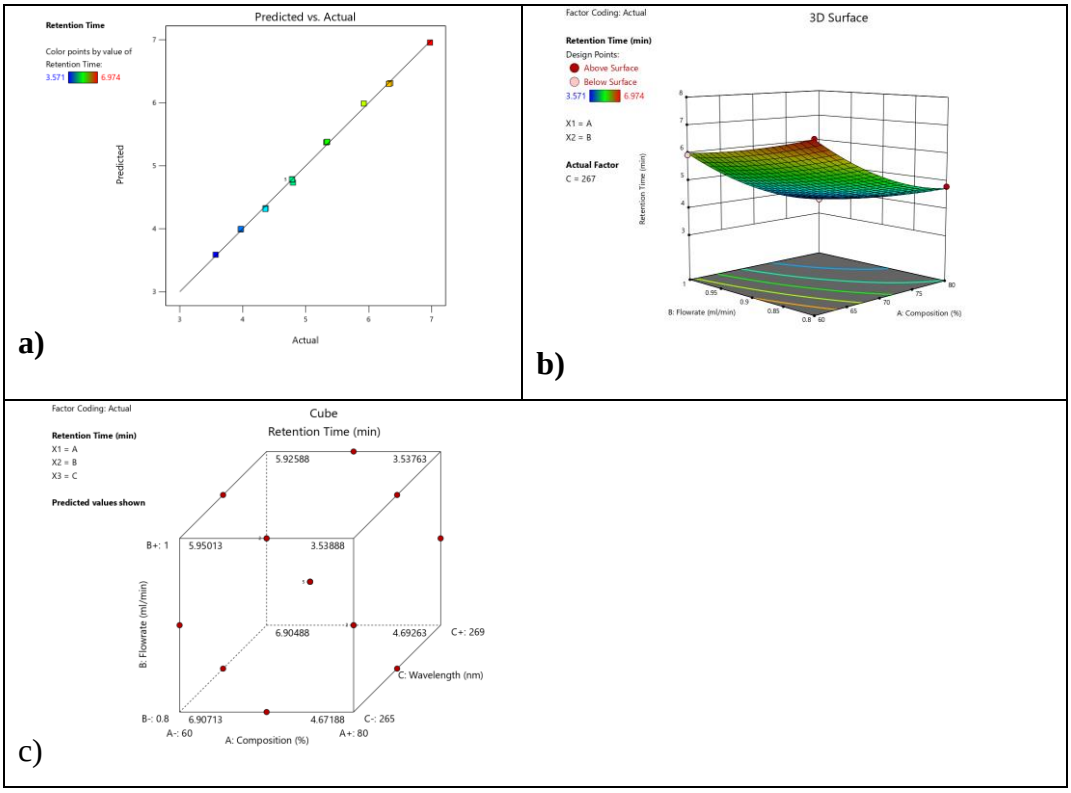


Figure 8: Retention time as response factor for ANOVA study a) Actual vs Predicted values, b) 3D Surface graph, c) Cube analysis for 3 variables and retention time

Peak Area plots:

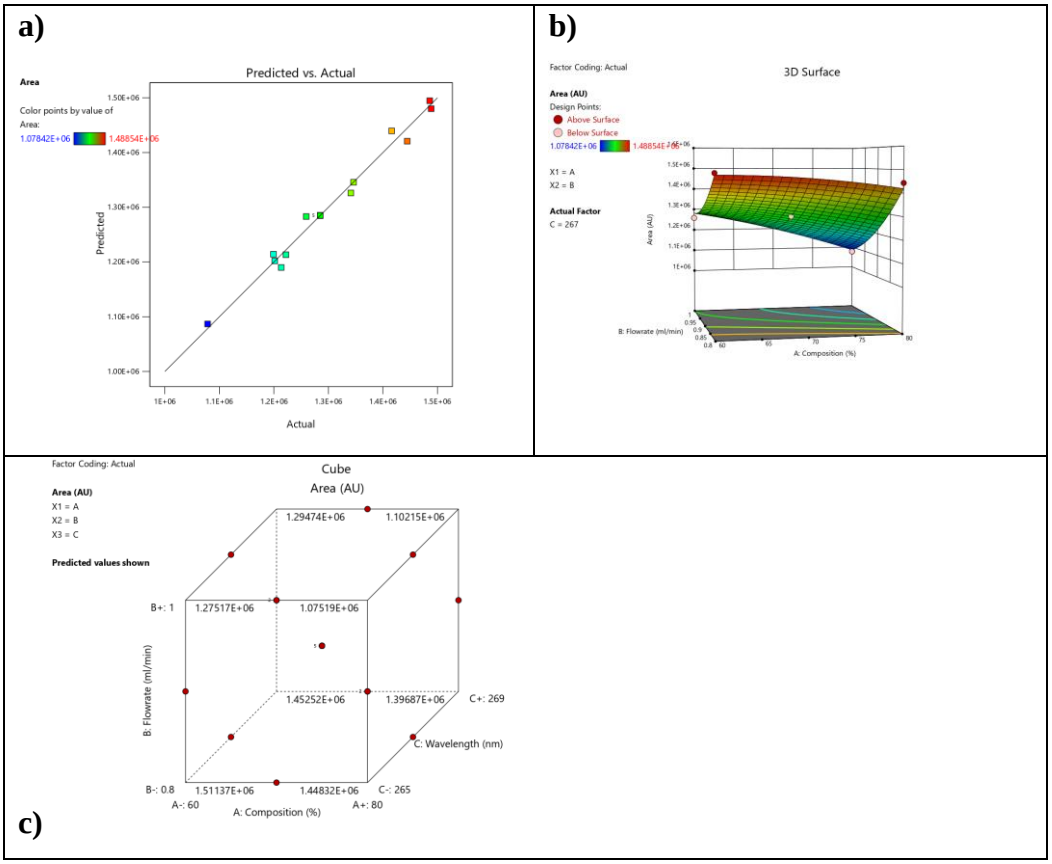
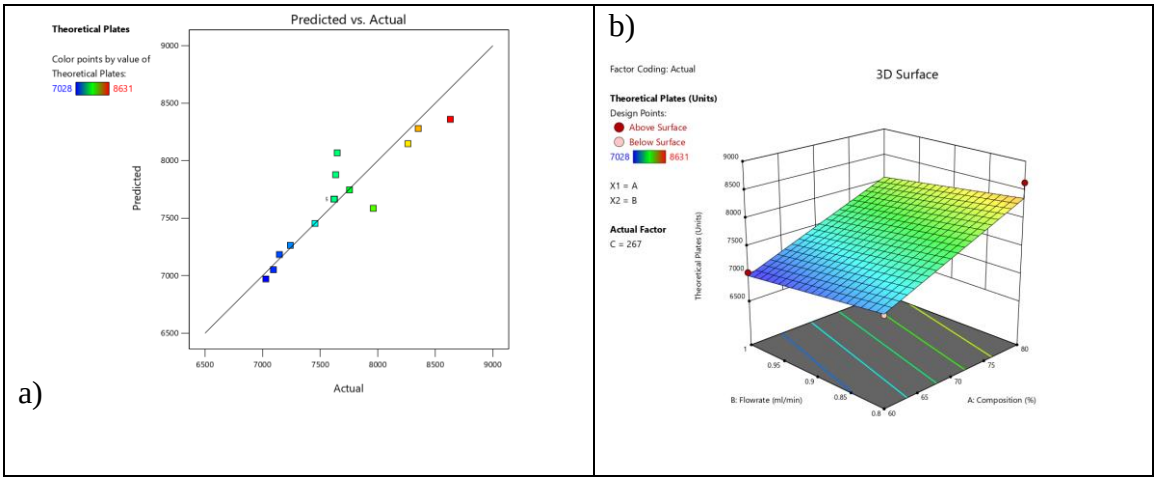


Figure: 9: Peak Area as response factor for ANOVA study a) Actual vs Predicted values, b) 3D Surface graph, c) Cube analysis for 3 variables and retention time.

Theoretical Plates response and plots:



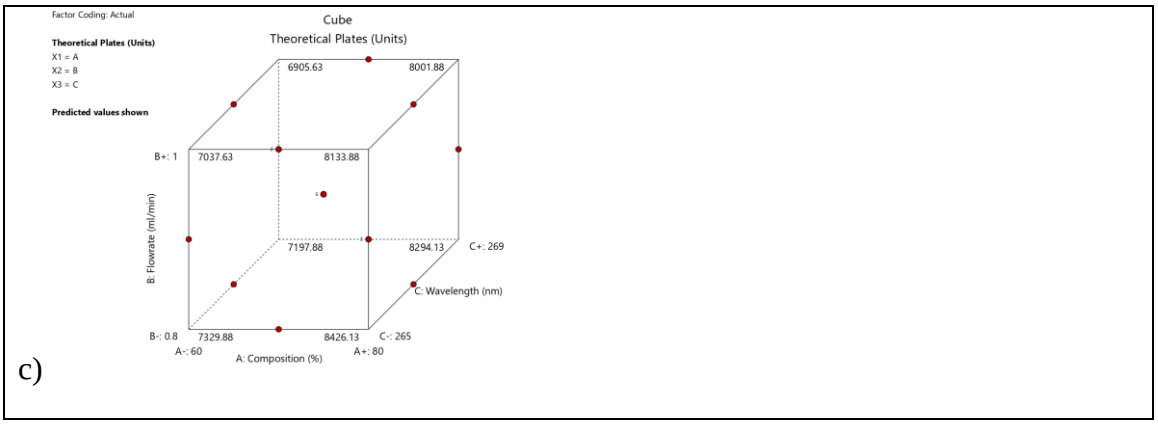


Figure 10: Theoretical Plate as response factor for ANOVA study a) Actual vs Predicted values, b) 3D Surface graph, c) Cube analysis for 3 variables and retention time.

Asymmetry as response and plots:

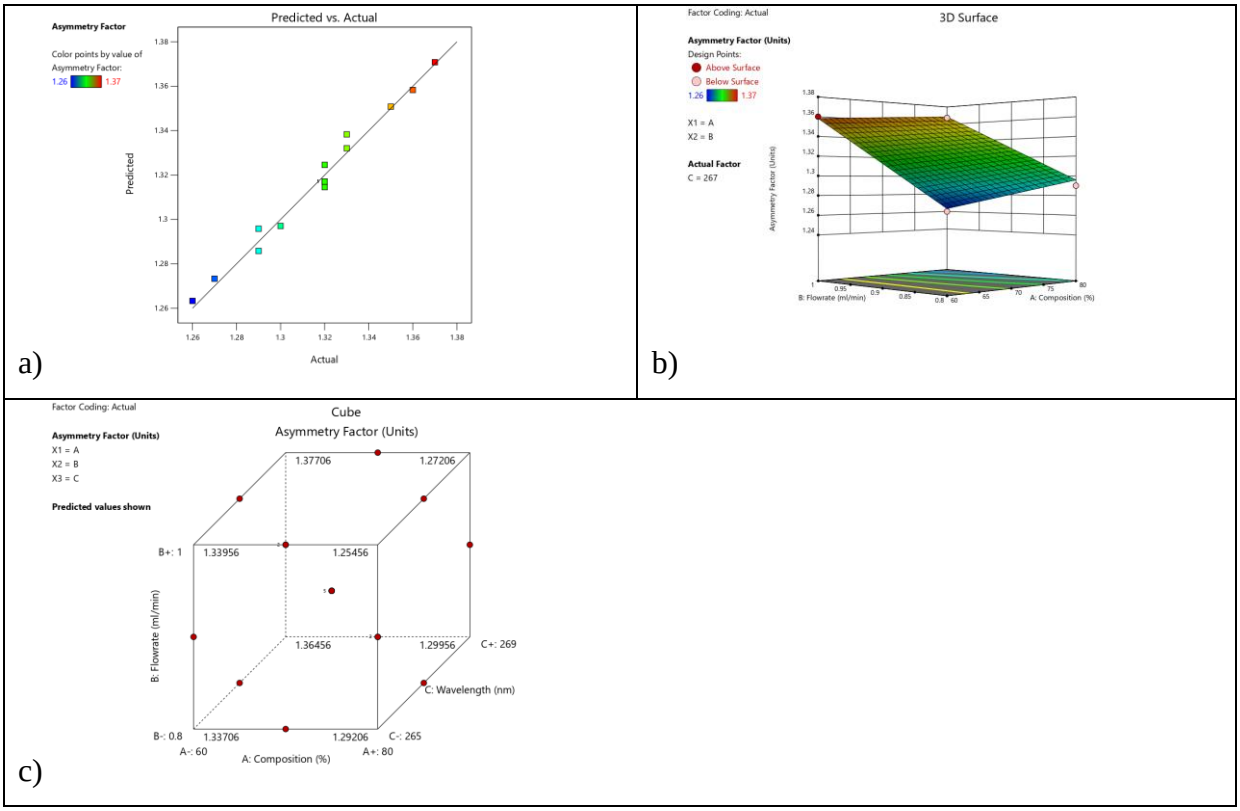


Figure 11: Asymmetry as response factor for ANOVA study a) Actual vs Predicted values, b) 3D Surface graph, c) Cube analysis for 3 variables and retention time.

Based on the development trails from design expert studies, trail 1 with high theoretical plates, short retention time and low asymmetry was selected for validation.

Percentage Assay study:

20 microliters of Alectinib sample and standard solutions were injected into three chromatographic systems. The peak area of each injection was measured. The concentration is calculated by comparing the peak area of the standard chromatogram with the sample chromatogram using the following formula:

The obtained results are shown in the table 3.

Table 23: Results of % of assay

Sr. No.	Conc.	Area of standard	Area of sample	% assay
1	30 ppm	911570	909135	99.7328784

System suitability parameters:

System Suitability Parameters are the standards to compare your results with the aproximate standard values. They includes as follows

1. Resolution: Resolution value should be greater than 1.75. This parameter is applicable only when there is a combination of two samples. In case of single sample it will show zero '0' value.

2. Theoretical Plates: Number of theoretical plates should be greater than 2000. It indicates the efficiency of column.

3. Tailing/Asymmetry Factor: Value of asymmetry factor should be less than 2.

All the above required values are already reported in the individual spectra, no need to calculate them.

Linearity:

Alectinib calibration standard solutions at concentrations of 10, 20, 30, 40 and 50 µg/ml were prepared and injected into the chromatographic system. Linear regression was used to plot the calibration curve of Alectinib peak area (y-axis) versus concentration (x-axis). Each peak area is used to calculate the correlation coefficient (r2) 8.9. The linear results are shown in Table 4 and Figure 3.

Table 24: Linearity levels preparation assay of Alectinib.

Concentration	Area
10	300949

20	615329
30	911570
40	1211079
50	1554117

The values of Conc. and Area in the given column and get the Linearity graph with R sq. value.

Limit: The 'R'sq. value should be near to 1

y=mx+c

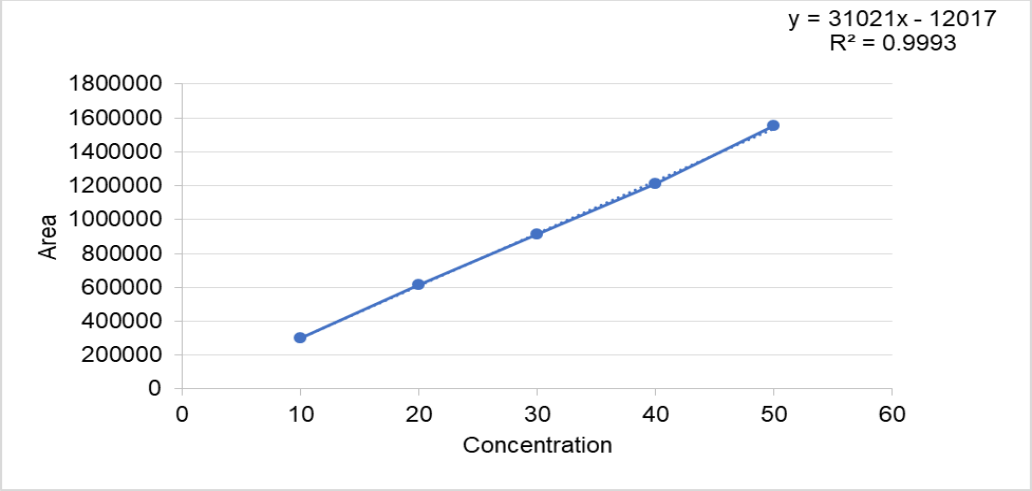


Figure 12: Calibration plot obtained for assay of Alectinib

Accuracy (recovery):

The accuracy of this method was determined by calculating the recovery value of Alectinib by the standard addition method. Specific volumes of 50, 100, and 150% Alectinib standard solution were added to the predetermined Alectinib sample solution and injected into the chromatographic system. Each standard solution was prepared and analyzed in triplicate. The peak area of each point was used to calculate the recovery rate. The results were done according to ICH guidelines. According to ICH guidelines, the recovery rate should be between 98-102%. The results are summarized in Table 25.

Table 25: Accuracy Data for Alectinib

Alectinib	Standard Deviation	Accuracy	Precision

Sr. No.	Conc.	Area	Mean	SD	% SD	% RSD
1	10	300949	301560.6667	702.5555	0.232973	
	10	301405				
	10	302328				
2	30	911570	916759	5903.533095	0.643957	0.215599733
	30	915525				
	30	923182				
3	50	1554117	1562968.333	8619.226029	0.551465	
	50	1571335				
	50	1563453				

Limit: %SD and %RSD value should be less than 2%

Limit of detection (LOD) and limit of quantification (LOQ):

The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 3.3 × SD/S and 10 × SD/S, respectively, according to ICH guidelines, where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the lowest analyte concentration that gives a measurable response (signal-to-noise ratio of 3). The LOQ is the lowest analyte concentration that gives a definite and measurable response (signal-to-noise ratio of 10). The calculated LOD and LOQ values are shown in Table 26.

$$LOD = 3.3 \times S / SD$$

and

$$LOQ = 10 \times S / SD$$

Table 26: Limit of detection (LOD) and limit of quantification (LOQ) data for Alectinib

Sr. No.	Drug	SD	Slope	LOD	LOQ
1	Alectinib	702.55	31021	0.074736952	0.22647561

Precision:

The accuracy of the Alectinib drug solution method was tested by increasing Six injection samples containing the same concentration of 100 µg/ml Alectinib drug solution prepared and injected into the chromatography system. The peak area of each injection was used to calculate percent RSD. To estimate the average precision, six injections with a concentration of 100 µg/ml Alectinib were analyzed on different days by different analysts using different columns of the

same parameters. Each injection area was used to calculate the % RSD is 0.189191818 % and 0.10682382 %. From the data obtained in Tables 7 and 8, the developed method was found to be accurate.

Table 27: Method precision for Alectinib drug solution (Interday)

Interday		**Area**	**Standard Deviation**		**Accuracy**	**Precision**
Sr. no.	**Conc.**		**Mean**	**SD**	**% SD**	**%RSD**
1	30	911570	916759	5903.533095	0.643956	0.189191818
	30	915525				
	30	923182				
2	30	987920	977727.333	5606.658526	0.911373	
	30	971413				
	30	973849				

Table 28: Method precision for Alectinib drug solution (Intraday)

Intraday		**Area**	**Standard Deviation**		**Accuracy**	**Precision**
Sr. no.	**Conc.**		**Mean**	**SD**	**% SD**	**%RSD**
1	30	911570	916759	5903.5330	0.64395693	0.10682382
	30	915525				
	30	923182				
2	30	9110393	9191603.667	7075.8800	0.79502862	
	30	9212367				
	30	9252051				

% Recovery: The average % recovery was found to be between 99-100% as shown in table 9.29

Table 29: Method % recovery for Alectinib

Sr. No.	**% Composition**	**Area of Standard (Area Units)**	**Area of Sample (Area Units)**	**% Recovery (%)**	**Conc. Taken (ppm)**	**Conc. Found (ppm)**
1	50% Recovery	911570	904672	99.2432	30	29.7729
2	100% Recovery	1211079	1202765	99.3135	40	39.7254
3	150% Recovery	1554117	1544895	99.4066	50	49.7033

Robustness: The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The Wavelength was changed by ± 2 nm proportion and the pH of mobile phase was changed in (±0.2) proportion of optimized chromatographic condition.

Table 30: Method robustness for Alectinib

	Conc.	**Area**	**Mean**	**SD**	**%SD**
Change in Wavelength	20	615329	612269.7	5632.98	0.9200164
	20	615711			
	20	605769			
Change in pH	20	615329	616042.0	4137.83	0.6716801
	20	612307			
	20	620490			

Ruggedness:

Standard preparation, stock preparation and sample preparation of Alectinib tablets were prepared according to the methodology given in Part IV. Samples were incubated with standard solutions under different chromatographic conditions as described below.

Table 31: Method of Ruggedness for Alectinib

Concentration	**Area**
10	321457
20	641511
30	961073
40	1309632
50	1605482

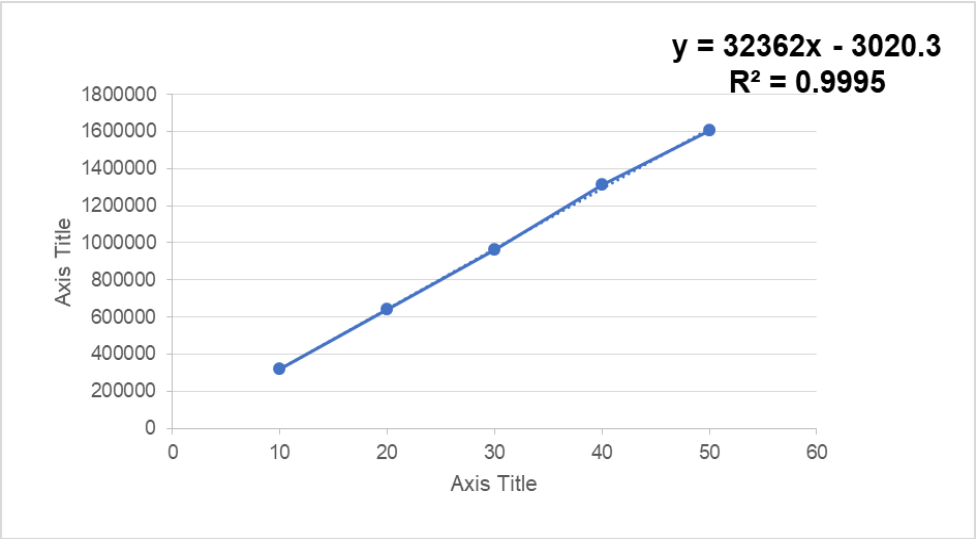


Figure 13: Calibration plot obtained for Ruggedness of Alectinib

Forced Degradation Study

Stress degradation for Alectinib was carried out and it showed degradation in every condition. It showed higher degradation in base hydrolysis with upto 23.64 % degradation and upto 17.04% degradation in Acidic condition. In the oxidation condition, heat degradation and Photolytic conditions the degradation was found to be 8.79%, 1.08% and 0.14% respectively.

Table No 32: Forced Degradation Study

Sr. No.	Degradation	Area of Standard	Area of degradation sample	Degraded upto %	Actual % degradation
1	Acid Degradation	1554117	1289239	82.9563	17.0436
2	Base Degradation	1554117	1186677	763569	23.6430
3	H2O2 Degradation	1554117	1417416	91.2039	8.796055
4	Photolytic Degradation	1554117	1551956	99.8609	0.139050
5	Thermal Degradation	1554117	1537233	98.9135	0.086406

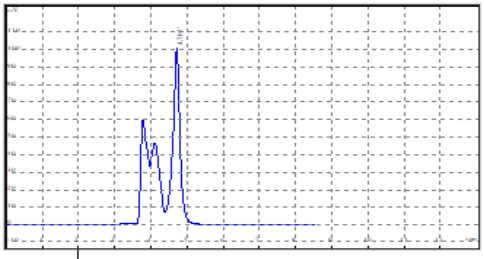


Fig. No 14: Acid Hydrolysis

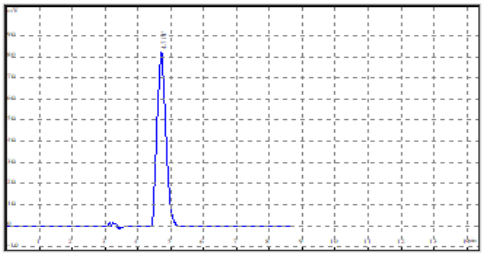


Fig. No 15: Base Hydrolysis

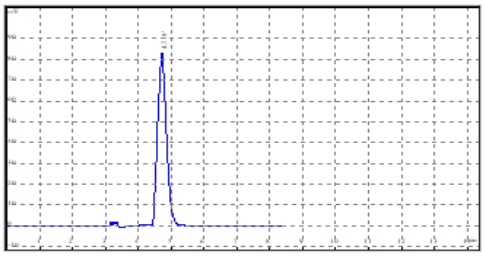


Fig. No 16: H2O2 Degradation

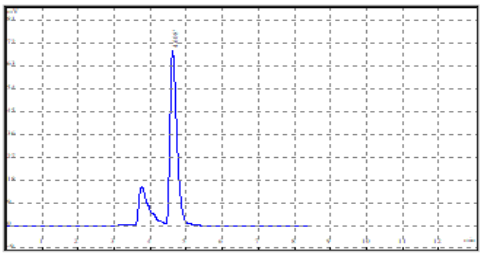


Fig. No 17: Photolytic Degradation

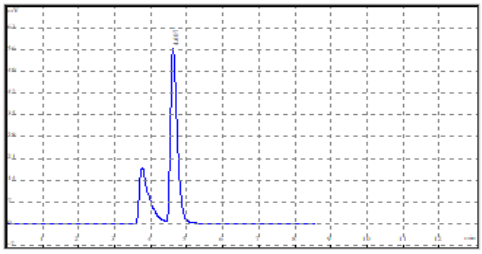


Fig. No 18: Thermal degradation

CONCLUSION:

An attempt has been made to Develop Reverse Phase High Performance Liquid Chromatographic method for the estimation of Alectinib and to validate the developed method according to ICH Q2 (R1) guidelines. The RP-HPLC method for the estimation of Alectinib was

developed. The quantification was carried out by using Cosmosil C18 (250 mm × 4.6 mm, 5 µm) as stationary phase, Methanol and water [80:20] having pH 3.0 as mobile phase. Mobile phase was maintained at a flow rate of 0.8 ml/min at 267 nm. The drug was eluted at 4.69 minutes. The Linearity range (µg/ml) was selected between 10-50 ppm, were Regression Equation (y = mx+c) was used to calculate the equation in which y is 31021x-12017. During the method validation the Correlation Coefficient (r^2) was found 0.999. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.0747 µg/mL and 0.2264 µg/mL, respectively. In the method development the % Recovery rate was found between 99-100 % which is satisfied as per ICH Q2 (R1) Guidelines. The study of Intra-Day and Inter-Day Precision (%RSD) were obtained 0.11% & 0.18 % respectively. The method provides selective quantification of Alectinib. This developed RP-HPLC method for estimation of Alectinib is accurate, precise, robust and specific. The drug was found to be degraded in stressed condition. The method has been found to be better than previously reported method, because of its less retention time, isocratic mode and use of an economical and readily available mobile phase, readily available column, UV detection and better resolution of peaks.

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