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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AVELUMAB AND AXITINIB

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ABSTRACT:

A Simple, Rapid, Precise, Sensitive and Reproducible Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method has been developed for the Quantitative analysis of Avelumab and Axitinib in Pharmaceutical dosage form. Chromatographic separation of Avelumab and Axitinib in was achieved on Water Alliance-e2695 by using Symmetry C18 (150x 4.6 ,3.5µm) column and the Mobile phase containing ACN an 0.1% TFA in the ratio of 60:40%v/v. The Flow rate was 1.0ml/min. Detection was carried out by Absorbance at 222nm using a Photodiode array detector at ambient temperature. The number of Theoretical plates and Tailing factor for Avelumab and Axitinib were NLT 2000 and should NMT 2 respectively. The Calibration curve range of Peak Areas %Relative Standard Deviation should be less than 2.According to ICH Guidelines the method was validated. The method was found to be Simple, precise, Accurate and Robust method for Quantitative Analysis of Avelumab and Axitinib study of its Stability.

Key words: RP-HPLC, ICH, Avelumab and Axitinib

Introduction : Avelumab, also known as Bavencio, is a fully human monoclonal antibody used to treat Merkel cell carcinoma, urothelial carcinoma and renal cell carcinoma. In January 2017, te European Medicines Agency (EMA) designated it as an orphan drug for the treatment of gastric cancer.Avelumab is used to treat a type of skin cancer called Merkel Cell carcinoma.

Pfizer created Axitinib, a small molecule tyrosine kinase inhibitor. The US Food and Drug Administration approved it for RCC. It works by blocking the action of an abnormal protein that signals cancer cells to multiply.

The Literature survey reveals that analytical and bioanalytical methods reported for the analysis of Axitinib. There are no methods were reported to simultaneous quantification of Avelumab and Axitinib in bulk and formulation. The goal of the present work is to develop and validate a Novel, Simple, Sensitive, Specific, Precise, Accurate and Robust RP-HPLC method for the determination of Avelumab and Axitinib in bulk and pharmaceutical dosage form. To Validate the Developed method As per ICH Guidelines.

MATERIALS AND METHODS:

Chemicals and Reagents: Merck (India) Ltd, Worli, Mumbai, India, Provided HPLC grade acetonitrile, Milli Q water, and ortho phosphoric acid. Both Axitinib and Avelumab APIs were obtained as reference standards from Zydus, Cadila and Ahmadabad.

Instrumentation: Water alliance e2695 chromatographic system consisting of quaternary pump, PDA detector 2996 and chromatographic Software Empower 2.0 was used..

Mobile Phase: Add Acetonitrile and 0.1%TFA in 60:440v/v ratio and mixed well and sonicated for 15min, filter with 0.45µ membrane filter paper is used as mobile phase

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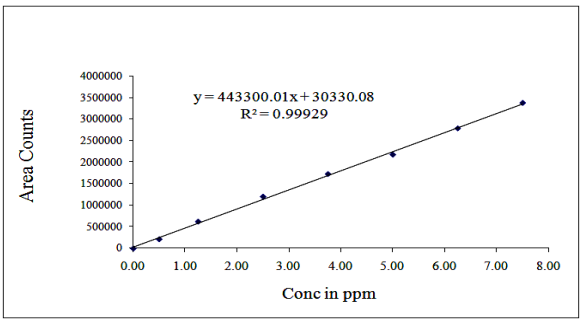
Preparation of standard solution: Working standards of 5 mg Axitinib and 20 mg Avelumab must be correctly weighed. These standards were put in a 100 mL volumetric flask, filled with 70 mL diluents, and sonicated for 10 minutes to dissolve the contents before being made up to the mark with the same diluents. Using the diluents, dilute 5 mL of the above solution to 50 mL.

Preparation of sample stock solution : In a 100 ml volumetric flask, measure correctly 5 mg equivalent weight of Axitinib and 20 mg equivalent weight of Avelumab sample. Add about 70 mL of diluents, sonicate for 30 minutes to fully dissolve the contents, and make up to the mark with diluents. Using a 0.45 syringe filter, filter the solution.
Optimized method chromatographic conditions were given below for the assay, these optimized conditions are used for the determination Avelumab and Axitinib drug in bulk and formulation. The chromatograms for the blank, standard and sample were showed below:

S. No.	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1%TFA (60:40)
2	Column	Symmetry C_{18} (150x4.6 mm, 3.5 µ)
3	Mode	Isocratic mode
4	Flow rate	0.8 ml/min
5	Column temperature	Room temperature
6	Sample temperature	Room temperature
7	Wave length	222 nm
8	Injection volume	10 µl
9	Run time	6 min

Linearity: Inject each level into the chromatographic system and measure peak area. Plot a graph of peak area vs concentration (on X-axis concentration and on y-axis peak area) and calculate the correlation coefficient. The response of the drug was found to be linear in the concentration range of 2µg/ml for Avelumab and 0.5-7.5 µg/ml for Axitinib and the correlation coefficient was 0.999.

Linearity	Axitinib		Avelumab	
	Conc. (µg/ml)	Area of analyte	Conc. (µg/ml)	Area of analyte
Linearity-1	0.50	365098	2.00	336524
Linearity-2	1.25	624785	5.00	915181
Linearity-3	2.50	1200156	10.00	1748692
Linearity-4	3.75	1726425	15.00	2544693
Linearity-5	5.00	2174715	20.00	3265524
Linearity-6	6.25	2784593	25.00	4030598
Linearity-7	7.50	3375144	30.00	5036529
Slope	443300.01		163895.36	
Intercept	30330.08		42617.13	
CC	0.99929		0.99927	



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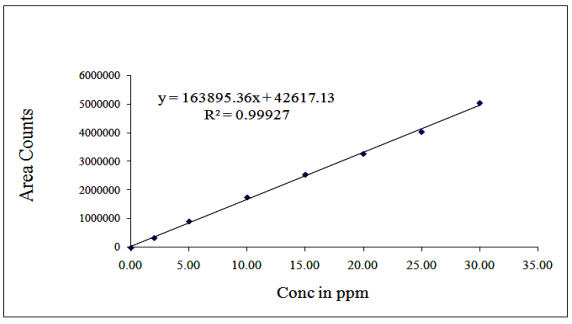

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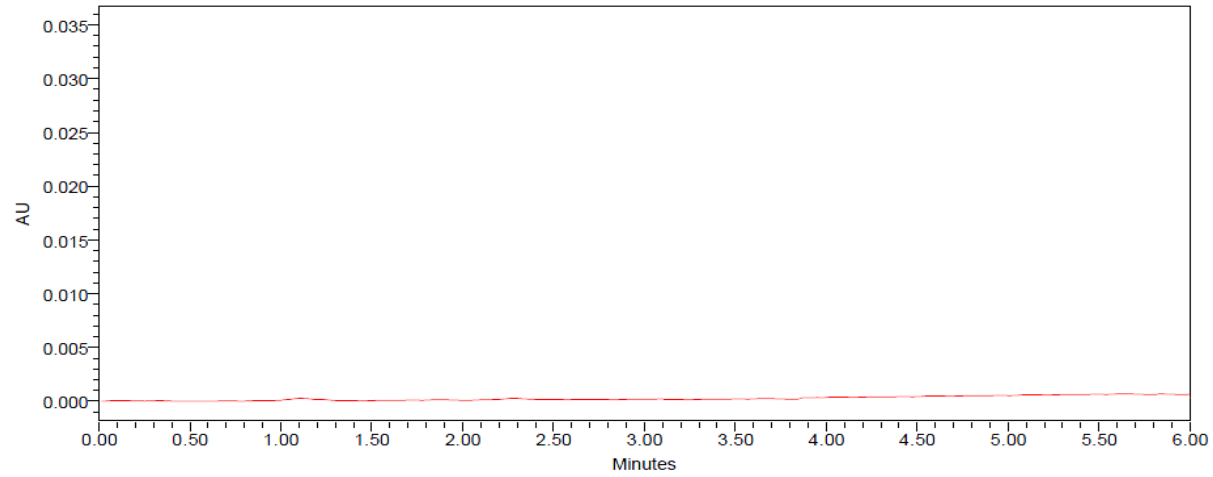
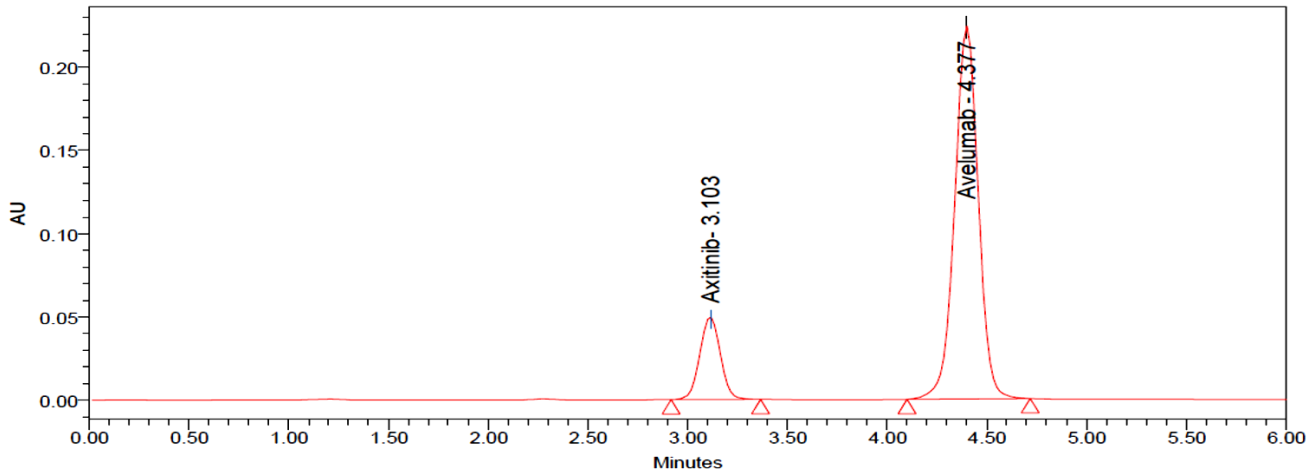
Figure 1: Linearity plot of (a)Axitinib and (b) Avelumab



Chromatogram of blank



Chromatogram of Standard

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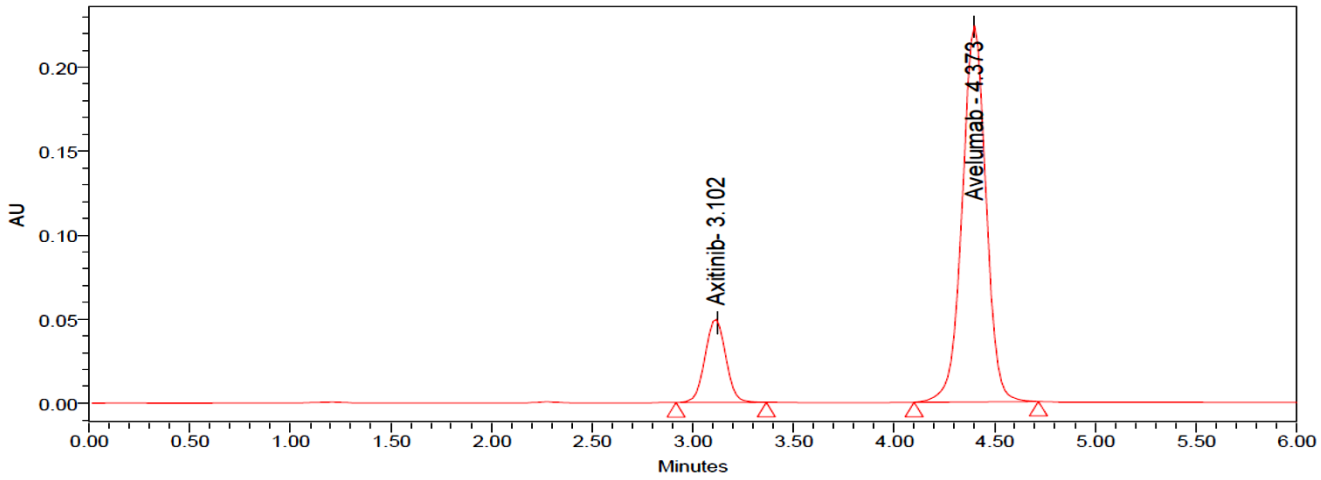

Chromatogram of sample

Result and Discussion:

The developed method was validated was validated according to ICH guidelines for the parameters like linearity, precision, accuracy, robustness, ruggedness, forced degradation and stability of the method was studied by the Avelumab and Axitinib.

Specificity

Specificity was the ability to assess unequivocally the analytic in the presence of components which may be expected to be present. Typically these include impurities, degrades, matrix etc. Placebo interference was checked for one strength in duplicate, equivalent to about the weight of placebo as per the test method. It was observed that there is no interference at retention time of Avelumab and Axitinib peaks.

Name of the solution	**Retention time**
Blank	No peak
Placebo	No peak
Avelumab	4.347
Axitinib	3.109

Accuracy:

The Accuracy of the method was determined by a known amount standard drug was added to fixed amount of pre-analyzed capsule solution with the spiking levels of 50%, 100% and 150%. Percentage recovery was calculated by comparing the area before and after addition of the standard drug. Recovery of Axitinib and Avelumab were determined at three different concentration levels. Inject each level into the chromatographic system. The mean recovery was 99.3-100.6%

S. No.	% Level	% Recovery	
		Axitinib	Avelumab
1	50	100.02	100.14
2	100	99.88	99.95
3	150	100.14	100.32

Precision:

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To study the system precision, six replicates of the standard of 20µg/ml of Avelumab and 5µg/ml of Axitinib were injected into the HPLC system. The system suitability parameters are evaluated and found to be within the limits. The %RSD of Avelumab was 0.91 and Axitinib was 0.55

S. No.	Area for Avelumab	Area for Axitinib
1	3256478	2136259
2	3214562	2154987
3	3232588	2136502
4	3225964	2165471
5	3250124	2145985
6	3296983	2156374
Average	3246117	2149263
Std dev	29310.92	11736.45
%RSD	0.91	0.55

Result of method precision

Intermediate precision (Ruggedness):

The intermediate precision of assay method was carried out by using the same capsule of Avelumab and Axitinib using two different systems by using different analyst using different column and analyzed. The RSD of the result was found to be less than 2%.

LOD and LOQ:

Limit of detection and limit of quantification of the drug was calculated by using following equation as per ICH guidelines. The Limit of detection and Limit of quantification were evaluated by serial dilution of Avelumab and Axitinib stock solution in order to determine signal to noise ratio 3:1 for LOD and 10:1 for LOQ. The concentration of LOD and LOQ for Avelumab and Axitinib were 0.025µg/ml and 0.0063µg/ml and 0.825µg/ml, 0.0206µg/ml.

Robustness:

This was evaluated by deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method. The result of the Robustness study unaffected on developed assay method.

System suitability:

System suitability was studied under each validation parameters by injecting six replicates of the standard solution. The system suitability parameters were shown below

System suitability parameter	Acceptance criteria	Drug name	
		Axitinib	Avelumab
% RSD	NMT 2.0	1.24	0.54
USP Tailing	NMT 2.0	1.02	1.06
USP plate count	NLT 2000	3471	7885

Degradation studies:

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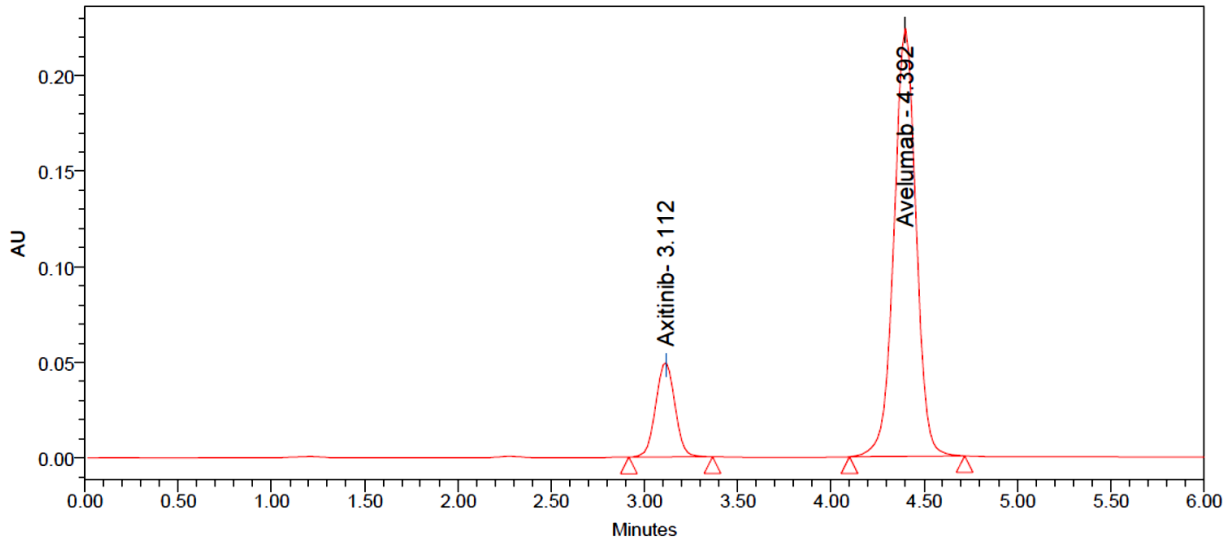
Forced degradation: In order to determine the analytical method and assay for the study stability indicating method in the formulation of Avelumab and Axitinib studied under various stress conditions to conduct forced degradation studies. Forced degradation such as acidic, basic, peroxide, hydrolysis, reduction, and thermal stress were studied in 0.1N to 1N conc. Levels.

Results: % Degradation results	Avelumab		Axitinib	
	Area	% Degradation	Area	% Degradation
Control	3245896	0.02	2154798	0.01
Acid	2746875	15.37	1859234	13.72
Base	2754102	15.15	1875124	12.98
Reduction	2813257	13.33	1820156	15.53
Hydrolysis	2789631	14.06	1846589	14.3
Peroxide	2865247	11.73	1864577	13.47
Thermal	2894012	10.84	1902342	11.72

Results of forced degradation



Chromatogram of control

Acid degradation: In 100ml of volumetric flask, measure correctly 5mg equivalent weight of Axitinib and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents and make up the volume up to the mark with diluents. Using 0.45 syringe filter, filter the solution. 1ml of sample is moved to a 10ml volumetric flask, along with 1ml of 1N HCl, and the mixture is left to sit for 15min. After 15min, apply 1ml of 1N NaOH and dilute to the desired dilents.

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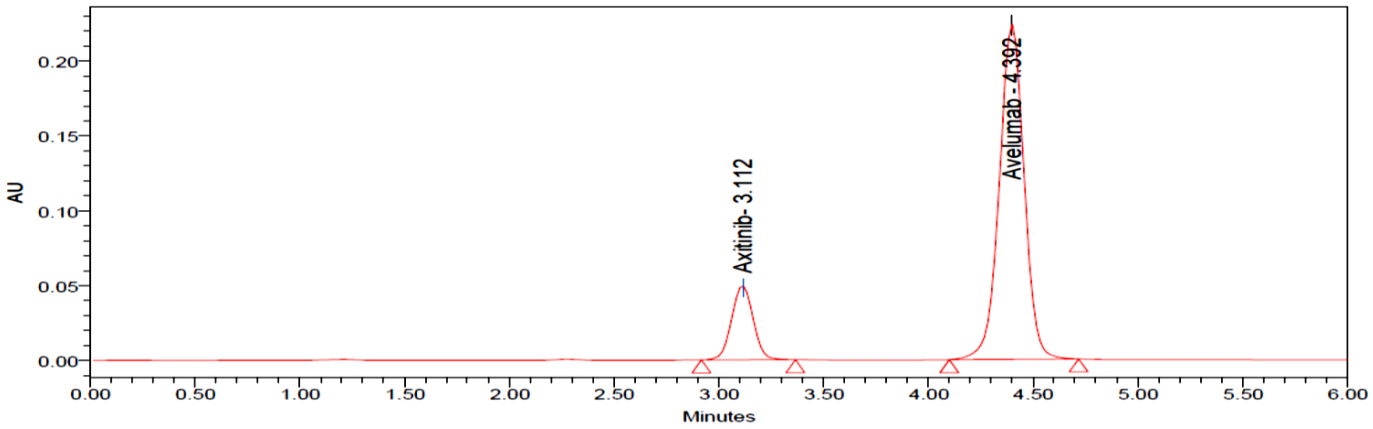
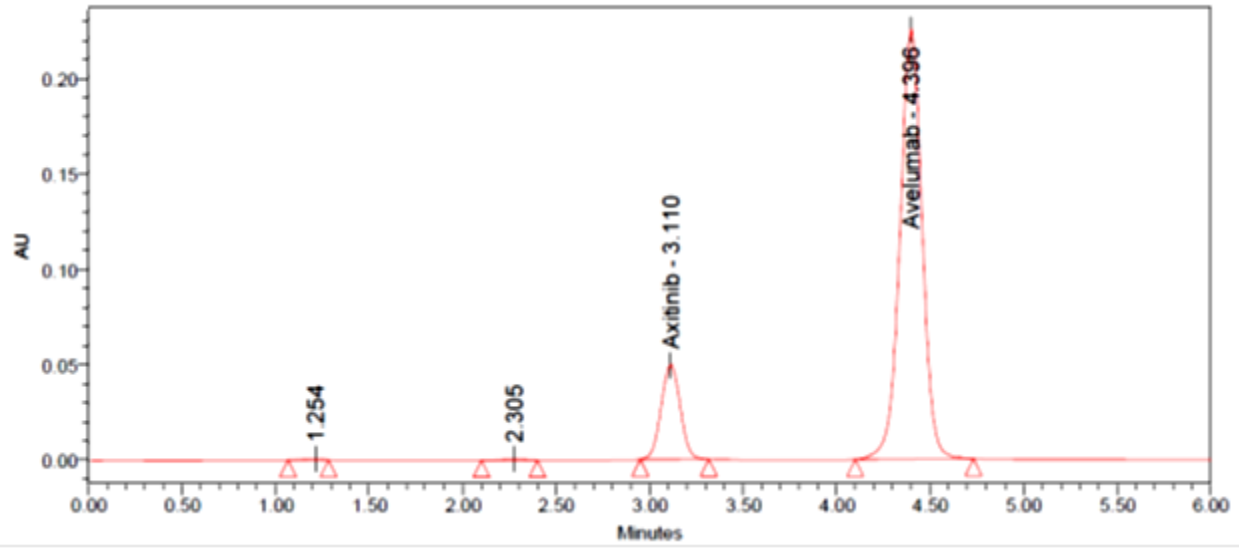


Chromatogram of acid degradation

Alkaline degradation: In 100ml volumetric flask, measure correctly 5mg equivalent weight of Axitinib and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents, and makeup the volume up to the mark with diluents. Using 0.45 syringe filter, filter the solution. 1ml of the sample is moved to 10ml volumetric flask, along with 1ml of 1N NaOH, and the mixture is left to sit for 15min. After 15min, apply 1ml of 1N HCl and dilute to the desired strength with diluents.



Chromatogram of alkali degradation

Peroxide degradation: In a 100ml volumetric flask, measure correctly 5mg equivalent weight of Axitinib and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents, and makeup the volume up to the mark with diluents. Using a 0.45 syringe filter the solution. 1ml of sample is moved to a 10ml volumetric flask, along with 0.3ml of 30% hydrogen peroxide and dilute to the desired strength with diluents.

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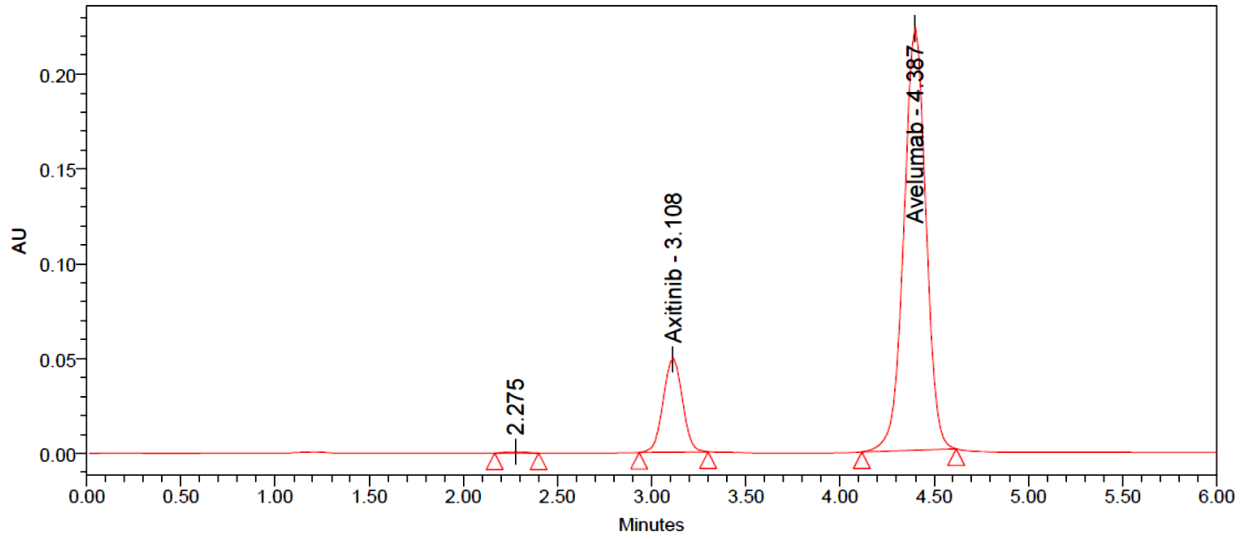
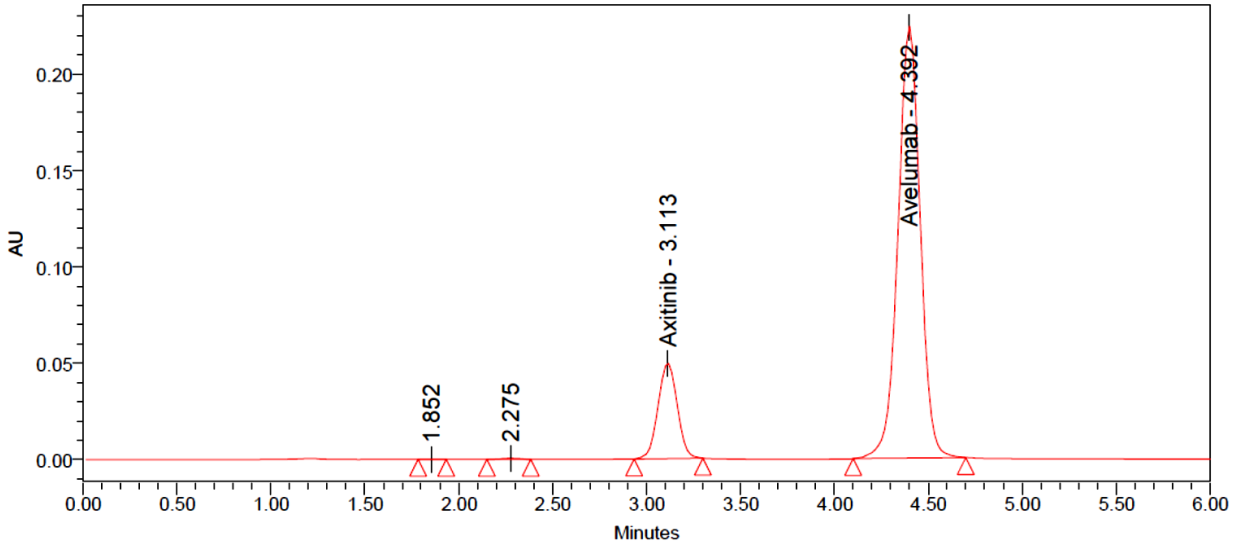


Chromatogram of peroxide degradation

Reduction degradation: In 100ml of volumetric flask, measure correctly 5mg equivalent weight of Axitinb and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents, and make up to the mark with diluents. Using a 0.45 syringe filter, filter the solution. 1ml of sample is moved to a 10ml volumetric flask, along with 1ml of 30% sodium bi sulphate solution and dilute to the desired strength with diluents.



Chromatogram of reduction degradation

Thermal degradation: In 100ml volumetric flask, measure correctly 5mg equivalent weight of Axitinib and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents, and make up the mark with diluents. Using a 0.45 syringe filter, filter the solution. The sample solution was placed in an oven at 105ºC for 6hrs. The resultant solution was into HPLC system.

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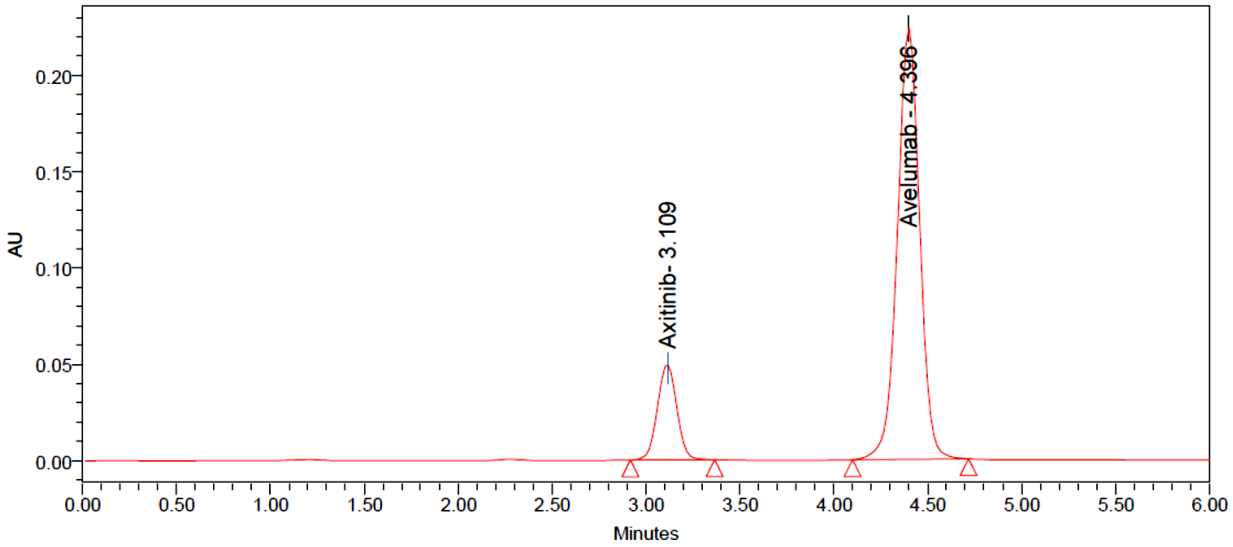
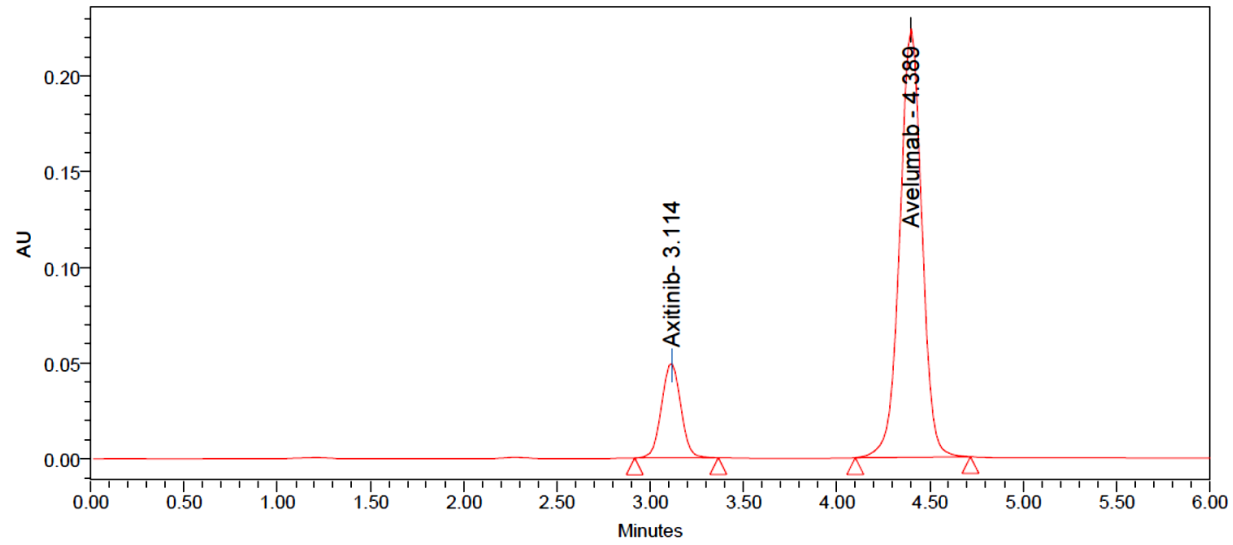


Chromatogram of thermal degradation

Hydrolysis degradation: In a 100ml volumetric flask, measure correctly 5mg equivalent weight of Axitinib and 20mg equivalent weight of Avelumab sample. Add about 70ml of diluents, sonicate for 30min to fully dissolve the contents, and make up to the mark with diluents. Using a 0.45 syringe filter , filter the solution 1ml of sample transferred into 10ml volumetric flask, add 1ml of water and made up to the mark with diluents.



Chromatogram of hydrolysis degradation

Conclusion:

The use of symmetry C18 column in this study resulted in better analyte elution with good resolution, increased plate count, and reduced tailing. As a result, C18 columns are frequently used to achieve high specificity in Axitinib and Avelumab studies in less time, as per ICH Q 3A(R2) guidelines. For simultaneous determination and quantification of Axitinib and Avelumab, the proposed method was found to be simple, precise, reliable, linear, robust, and fast. The sample recovery was consistent with their respective label statements, implying that there was no intervention in estimation.

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