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Eco Friendly and Economical Aqbd Analytical Method Development and Validation of Ramucirumab in Bulk and Injectable Dosage Form by Using RP-UPLC

Sabbavarapu Santhi Bhushan[1], Chennu M M Prasada Rao[2]
[1]Research Scholar, School of Pharmacy, Raffles university, Neemrana- 301705.
[2]Professor, School of Pharmacy, Raffles University, Neemrana- 301705.

Abstract
An Easy, sensitive, specific, and precise RP-UPLC method for the pharmaceutical dose estimation of Bevacizumab in injectable dosage form. Chromatogram was run through ACQUITY UPLC HSS C18 Column, 130A, 1.7 um, 2.1 mm X 100 mm. Mobile phase containing 0.1% OPA: Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 0.3ml/min. Temperature was maintained at 30.7°C. Optimized wavelength selected was 215.0nm. Retention time of Bevacizumab was found to be 0.945 min. %RSD of the Bevacizumab were and found to be 0.8. %RSD of Method precision of Bevacizumab was found to be 0.2 %Recovery was obtained as 99.75% for Bevacizumab. LOD, LOQ values obtained from regression equation of Bevacizumab were 0.05, 0.16. Regression equation of Bevacizumab is y = 11549x + 1531.9. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.
Key Words: *Bevacizumab, RP-UPLC, validation QbD software, method development, degradation, ICH Guidelines , Eco friendly analytical method development*

INTRODUCTION

Due to the various advantages such as speed, specificity, consistency, accuracy, precision, and ease of automation in these methods, most of the drugs in multicomponent dosage form can be analysed by the UPLC system. The UPLC approach avoids repetitive processes for extraction and isolation. In UPLC, there are distinct modes of differentiation. They are Normal Phase Mode, Inverted Phase Mode, Chromatography of Reversed Phase Ion Phase, Chromatography of Affinity and Chromatography of Size Exclusion.

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence Analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data (1) . Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.

The wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories. Different physico-chemical methods (1) are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis. The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements. [2-3]

- The analysis should take a minimal time.
- The accuracy of the analysis should meet the demands of Pharmacopoeia.
- The analysis should be economical.
- The selected method should be precise and selective.

Bevacizumab is a monoclonal anti-vascular endothelial growth factor antibody used in combination with antineoplastic agents for the treatment of many types of cancer.

CAS Number	:	216974-75-3
Protein Weight	:	Average: 149000.0 Da
Protein Formula	:	$C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$
Physical State	:	Liquid
Solubility	:	Water Solubility (25mg/mL)
Melting Point (°C)	:	61-71

Indication: As a vascular endothelial growth factor (VEGF) inhibitor, bevacizumab is used in several chemotherapy regimens to treat metastatic colorectal cancer; metastatic, unresectable, locally advanced or recurrent non-squamous non-small cell lung cancer etc

Pharmacodynamics: Bevacizumab binds circulating vascular endothelial-derived growth factor (VEGF) and blocks it from binding to its associated receptors, effectively blunting downstream signaling. The effects of bevacizumab have been shown to re-establish normal vasculature at the tumor site resulting in increased nutrient and oxygen supply, while also improving the delivery of chemotherapeutic drugs to the target area.

Mechanism of action: Transcription of the VEGF protein is induced by 'hypoxia inducible factor' (HIF) in a hypoxic environment. When circulating VEGF binds to VEGF receptors (VEGFR-1 and VEGFR-2) located on endothelial cells, various downstream effects are initiated. It should be noted that VEGF also binds to the neuropilin co-receptors (NRP-1 and NRP-1), leading to enhanced signaling.[4]

Experimental Work: [5-7]

Materials:

- Bevacizumab pure drug (API), Bevacizumab injection, Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instruments:

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises
- WATERS ACQUITY UPLC SYSTEM equipped with Binary pumps, TUV detector and Auto sampler integrated with Empower 2 Software.
- UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Bevacizumab solution.

METHODOLOGY [8-21]

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of buffer:

0.1% OPA Buffer: Take 1ml of 0.1% OPA into 1000ml beaker and add water milli of hplc grade and sonicate.

Preparation of Standard stock solutions: Accurately weighed 12.5mg of Bevacizumab transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (250µg/ml solution of Bevacizumab)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (25µg/ml of Bevacizumab).

Preparation of Sample stock solutions: 1 vial of injection was transferred into a 100 ml volumetric flask, 3/4thml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by UPLC filters (250µg/ml of Bevacizumab)

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (25µg/ml of Bevacizumab)

Validation:

System suitability parameters:

The system suitability parameters were determined by preparing standard solution of Bevacizumab (25ppm) and the solution were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision

Preparation of Standard stock solutions: Accurately weighed 12.5mg of Bevacizumab transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (250µg/ml solution of Bevacizumab)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (25µg/ml of Bevacizumab).

Preparation of Sample stock solutions: 1 vial of injection was transferred into a 100 ml volumetric flask, 3/4thml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by UPLC filters (250µg/ml of Bevacizumab)

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (25µg/ml of Bevacizumab)

The test solution were determined by preparing test solution of Bevacizumab (25ppm) and the solution were injected six times and the % RSD for the area of six standard injections results should not be more than 2%.

Linearity:

Preparation of Standard stock solutions: Accurately weighed 12.5mg of Bevacizumab transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (250µg/ml solution of Bevacizumab)

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (6.25µg/ml of Bevacizumab)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Bevacizumab)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (18.75µg/ml of Bevacizumab)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (25µg/ml of Bevacizumab)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (31.25µg/ml of Bevacizumab)

150% Standard solution: 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml (37.5µg/ml of Bevacizumab)

Accuracy:

Preparation of Sample stock solutions: 1 vial of injection was transferred into a 100 ml volumetric flask, 3/4thml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by UPLC filters (250µg/ml of Bevacizumab)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria:
The % Recovery for each level should be between 98.0 to 102%
Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.
Robustness conditions like Flow minus (0.2ml/min), Flow plus (0.4ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35 °C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.
LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Bevacizumab, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents
LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each for Bevacizumab, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.
Degradation studies:[22-25]

Oxidation:
To 1 ml of stock solution of Bevacizumab, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For UPLC study, the resultant solution was diluted to obtain 25µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:
To 1 ml of stock solution Bevacizumab, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c. For UPLC study, the resultant solution was diluted to obtain 25µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:
To 1 ml of stock solution Bevacizumab, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. For UPLC study, the resultant solution was diluted to obtain 25µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:
The standard drug solution was placed in oven at 105°C for 6h to study dry heat degradation. For UPLC study, the resultant solution was diluted to obtain 25µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.
Photo Stability studies:
The photochemical stability of the drug was also studied by exposing the 250µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 40µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:
Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60°. For UPLC study, the resultant solution was diluted to obtain 25µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

6. RESULTS AND DISCUSSION
Solubility of Bevacizumab

Table 1 Solubility of Bevacizumab

S. No	Name of the Solvent	Bevacizumab
1	Water	Soluble
2	Methanol	Soluble
3	Ethanol	Soluble
4	phosphate-buffered saline.	Highly soluble
5	Acetonitrile	Highly soluble

UV Spectrum of Bevacizumab

The UV Spectrum of Bevacizumab in the methanol at 215 nM .The figure mentioned in Figure 1

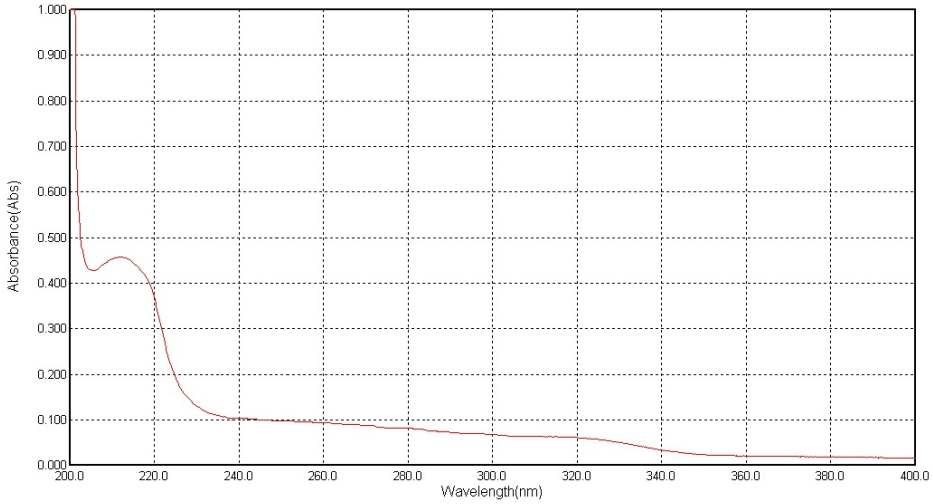


Figure 1 UV Spectrum of Bevacizumab

Initial HPLC runs of Bevacizumab

Initial HPLC runs of Bevacizumab of 25µg/ml concentration were performed using

- Different buffer viz, Potassium dihyrogen ortho phosphate and Ortho phosphoric acid.
- Different organic modifier viz, acetonitrile and methanol
- Different columns such as ACQUITY UPLC HSS PFP Columns, ACQUITY Premier Glycoprotein BEH Amide Column, 300Å, 1.7 µm, 2.1 mm x 150 mm, ACQUITY Premier ACQUITY UPLC Glycoprotein BEH Amide Column, 300Å, 1.7 µm, 2.1 mm X 150 mm, 1/pk, Agilent C18 (150×4.6 mm, 5 µm), ACQUITY UPLC Glycoprotein BEH Amide Column, 300Å, 1.7 µm, 2.1 mm X 50 mm, and ACQUITY UPLC Peptide HSS T3 Column, 100Å, 1.8 µm, 1 mm X 100 mm.

Table 2 Design summary of CCD

Design Summary					
File version: DX 13.0.0 Study Type: Response surface Design Type: central composite design Design Model: Quadratic			ATP: Robustness CQA: Retention time, Tf, Theoretical plates Runs: 20		
CMPs	Unit	Type	Subtype	Min.	Max.
temperature	^{0}C	Numeric	Continuous	24.95	35.05
Flow rate	ml/min	Numeric	Continuous	0.2495	0.3505
%buffer ratio	%	Numeric	Continuous	31.59	48.41

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Total Three factors viz; % Organic concentration, Flow rate and column temperature were needed to be optimized. So, CCD was used to optimize these parameters which were varied over three level (high, mid, and low). different ranges of four parameters ranging from 31.59-48.41% of Buffer, column temperature 24.95 – 35.05^{0}C and 0.24-0.35 ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, tailing factor and Theoretical plates were generated. A desirability function applied to the optimized conditions to predict Retention Time, Tailing factor and Theoretical plates

Table 3. Central-composite model experimental design matrix with response

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std	Run	A: Flow Rate	B: Mobile Phase	C: Temperature	Retention Time	No of Theoretical Plates	Tailing Factor
		ml/min	%	0 C	min	num	num
1	11	0.27	35	27	1.061	2725	1.39
2	20	0.33	35	27	0.999	2672	1.37
3	12	0.27	45	27	0.98	2763	1.61
4	4	0.33	45	27	0.922	2703	1.59
5	6	0.27	35	33	1.029	2698	1.42
6	8	0.33	35	33	0.957	2644	1.47
7	19	0.27	45	33	0.941	2764	1.61
8	13	0.33	45	33	0.885	2683	1.71
9	10	0.249546	40	30	1.014	2708	1.47
10	7	0.350454	40	30	0.909	2599	1.54
11	2	0.3	31.591	30	1.056	2720	1.34
12	14	0.3	48.409	30	0.917	2798	1.72
13	5	0.3	40	24.9546	0.969	2653	1.46
14	16	0.3	40	35.0454	0.929	2631	1.6
15	3	0.3	40	30	0.947	2546	1.47
16	17	0.3	40	30	0.947	2563	1.48
17	9	0.3	40	30	0.954	2556	1.49
18	18	0.3	40	30	0.955	2554	1.5
19	15	0.3	40	30	0.955	2553	1.49
20	1	0.3	40	30	0.956	2551	1.48

Data analysis:

Three responses i.e. Retention time (Y1), No of theoretical Plates (Y2) and Tailing factor (Y3) were selected for statistical optimization and fitted to linear, Two factorial interactive (2FI) and quadratic models. The summary of statistics of Bevacizumab are presented in Table 4 and the comparative R2, adjusted R2, predicted R2, PRESS, s.d., F-values and p-values were determined using the Design Expert. A suitable polynomial model for describing the data was selected based on correlation (R2) and PRESS values. Response Y1, response Y2 and response Y3 followed linear model for Bevacizumab. Hence these models were selected for further optimization.

Table 4 Summary of model statistics for responses Y1, Y2 and Y3 for Bevacizumab

Model	R^2	AdjustedR 2	Predicted R2	PRESS	s.d.	F-value	p-value	Remarks
Response Y1 (Retention Time)								
Linear	0.9263	0.9125	0.8809	0.0050	0.0139	67.01	< 0.0001	
2FI	0.9277	0.8943	0.8038	0.0083	0.0153	0.0833	0.9679	
Quadratic	**0.9915**	**0.9838**	**0.9475**	**0.0022**	**0.0060**	**24.95**	**< 0.0001**	**Suggested**
Response Y2 (Number of theoretical Plates)								
Linear	0.1687	0.0128	-0.1803	1.494E+05	81.09	300.61	< 0.0001	
2FI	0.1716	-0.2108	-1.2234	2.814E+05	89.80	411.89	< 0.0001	
Quadratic	**0.9933**	**0.9873**	**0.9565**	**5510.83**	**9.21**	**4.34**	**0.0666**	**Suggested**
Response Y3 (Tailing Factor)								
Linear	0.9118	0.9015	-00130	0.3014	0.1046	132.01	<0.0001	**Suggested**
2FI	0.4538	0.2017	-0.9275	0.5734	0.1118	7.45	0.1141	
Quadratic	0.5983	0.2368	-3.1055	1.22	0.1093	6.45	1.2214	

The results of the Box-Behnken response fitting in the form of ANOVA for Bevacizumab given in the table 5 these Parameters were used to construct the independent variable response.

Table 5 ANOVA for the response of the Bevacizumab

Source	SS	df	MS	F- Value	p-Value
Response Y1 (Retention Time)					
Linear Model	0.0417	9	0.0046	129.25	< 0.0001
A- Flow rate	0.0132	1	0.0132	368.31	< 0.0001
B- Mobile Phase organic %	0.0223	1	0.0223	621.99	< 0.0001
C- Temperature	0.0035	1	0.0035	96.44	< 0.0001
AB	0.0001	1	0.0001	1.40	0.2649
AC	8.000E-06	1	8.000E-06	0.2232	0.6467
BC	5.000E-07	1	5.000E-07	0.0140	0.9083
A^2	0.0003	1	0.0003	7.62	0.0201
B^2	0.0025	1	0.0025	69.98	< 0.0001
C^2	6.175E-08	1	6.175E-08	0.0017	0.9677
Residual	0.0004	10	0.0000		
Total	0.0420	19			
Response Y_2 (Number of Theoretical Plates)					
Linear Model	1.257E+05	9	13966.38	164.74	< 0.0001

A- Flow rate	13621.95	1	13621.95	160.68	< 0.0001
B- Mobile Phase organic %	6819.63	1	6819.63	80.44	< 0.0001
C- Temperature	902.18	1	902.18	10.64	0.0085
AB	144.50	1	144.50	1.70	0.2209
AC	60.50	1	60.50	0.7136	0.4180
BC	162.00	1	162.00	1.91	0.1970
A^2	20955.58	1	20955.58	247.18	< 0.0001
B^2	82001.48	1	82001.48	967.26	< 0.0001
C^2	16725.09	1	16725.09	197.28	< 0.0001
Residual	847.77	10	84.78		
Total	1.265E+05	19			
Response Y_3 (Tailing Factor)					
Linear Model	0.1990	9	0.0221	150.89	< 0.0001
A- Flow rate	0.0038	1	0.0038	25.91	0.0005
B- Mobile Phase organic %	0.1668	1	0.1668	1137.75	< 0.0001
C- Temperature	0.0173	1	0.0173	117.74	< 0.0001
AB	0.0003	1	0.0003	2.13	0.1749
AC	0.0045	1	0.0045	30.79	0.0002
BC	0.0000	1	0.0000	0.0853	0.7762
A^2	0.0006	1	0.0006	4.17	0.0684
B^2	0.0034	1	0.0034	23.18	0.0007
C^2	0.0034	1	0.0034	23.18	0.0007
Residual	0.0015	10	0.0001		
Total	0.2005	19			

* Significant ($p<0.05$), SS: Sum of squares; MS: Mean sum of squares

The F-Values for the responses, Retention Time (Y1), Number of theoretical Plates (Y2), Tailing Factor (Y3) were found to be 119.58, 116.47 and 123.73 respectively, which will indicated that the model. The values of the Prob>F (< 0.05) for all responses indicated the significance if the models.

The goodness of fit model was checked y the coefficient of determination (R^2). The R2 values of the Retention Time (Y1), Number of theoretical Plates (Y2), Tailing Factor (Y3) were found to be 0.9171, 0.9607 & 0.9118 respectively. Based on these results indicated that the good correlation between the independent and dependent variables. The model was found to be significant with respect to the adjusted coefficient of determination (Adj R^2> 0.9000) values for the three dependent parameters. In all the cases Predicted R^2 values were in reasonable agreement with the Adj R^2 values.

The application of the central composite design yielded the following regression equation which is empirical relation ship

Retention time:
+0.9522 -0.0311A -0.0404B -0.0159 C +0.0025AB -0.0010 AC-0.0003BC+0.0044A^2 0.0132B^2 - 0.0001C^2
Number of theoretical Plates:
+2553.39 -31.58 A +22.35 B -8.13 C -4.25 AB -2.75 AC +4.50 BC +38.13 A^2+75.43B^2 +34.07 C^2
Tailing Factor:
+1.49 +0.0167 A+0.1105B+0.0355C+0.0062 AB+0.0237AC-0.0013BC+0.0065A^2+0.0154B^2 +0.0154C^2

Table 6 Confident interval response with the dependent and independent variables of Ritodrine

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Response Y1 (Retention Time)						
Intercept	0.9522	1	0.0024	0.9467	0.9576	
A-FR	-0.0311	1	0.0016	-0.0347	-0.0275	1.0000
B-MP	-0.0404	1	0.0016	-0.0440	-0.0368	1.0000
C-Temp	-0.0159	1	0.0016	-0.0195	-0.0123	1.0000
AB	0.0025	1	0.0021	-0.0022	0.0072	1.0000
AC	-0.0010	1	0.0021	-0.0057	0.0037	1.0000
BC	-0.0003	1	0.0021	-0.0050	0.0045	1.0000
A^2	0.0044	1	0.0016	0.0008	0.0079	1.02
B^2	0.0132	1	0.0016	0.0097	0.0167	1.02
C^2	-0.0001	1	0.0016	-0.0036	0.0034	1.02
Response Y_2(Number of Theoretical Plates)						
Intercept	2553.39	1	3.76	2545.02	2561.76	
A-FR	-31.58	1	2.49	-37.13	-26.03	1.0000
B-MP	22.35	1	2.49	16.79	27.90	1.0000
C-Temp	-8.13	1	2.49	-13.68	-2.58	1.0000
AB	-4.25	1	3.26	-11.50	3.00	1.0000
AC	-2.75	1	3.26	-10.00	4.50	1.0000
BC	4.50	1	3.26	-2.75	11.75	1.0000
A^2	38.13	1	2.43	32.73	43.54	1.02
B^2	75.43	1	2.43	70.03	80.84	1.02
C^2	34.07	1	2.43	28.66	39.47	1.02
Response Y_3 (Tailing Factor)						
Intercept	1.49	1	0.0049	1.47	1.50	
A-FR	0.0167	1	0.0033	0.0094	0.0240	1.0000
B-MP	0.1105	1	0.0033	0.1032	0.1178	1.0000
C-Temp	0.0355	1	0.0033	0.0282	0.0428	1.0000
AB	0.0062	1	0.0043	-0.0033	0.0158	1.0000
AC	0.0237	1	0.0043	0.0142	0.0333	1.0000

BC	-0.0013	1	0.0043	-0.0108	0.0083	1.0000
A^2	0.0065	1	0.0032	-0.0006	0.0136	1.02
B^2	0.0154	1	0.0032	0.0082	0.0225	1.02
C^2	0.0154	1	0.0032	0.0082	0.0225	1.02

SE: Standard error; CI: Confidence interval;
VIF: Variance of inflation factor
* Significant ($p<0.05$)
** Not significant ($p>0.05$)

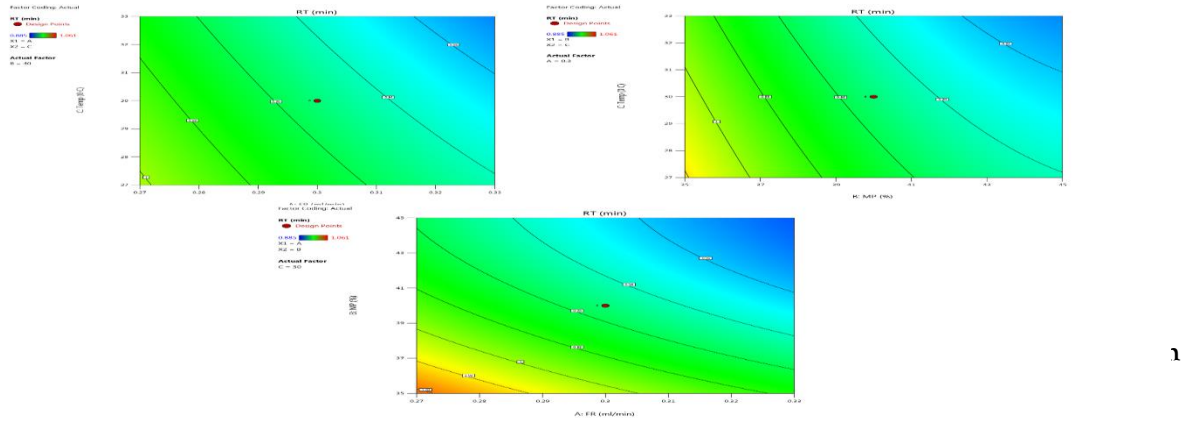


Figure3 3D Counter Plate of effect of column temperature and Mobile phase organic % Composition on the retention time

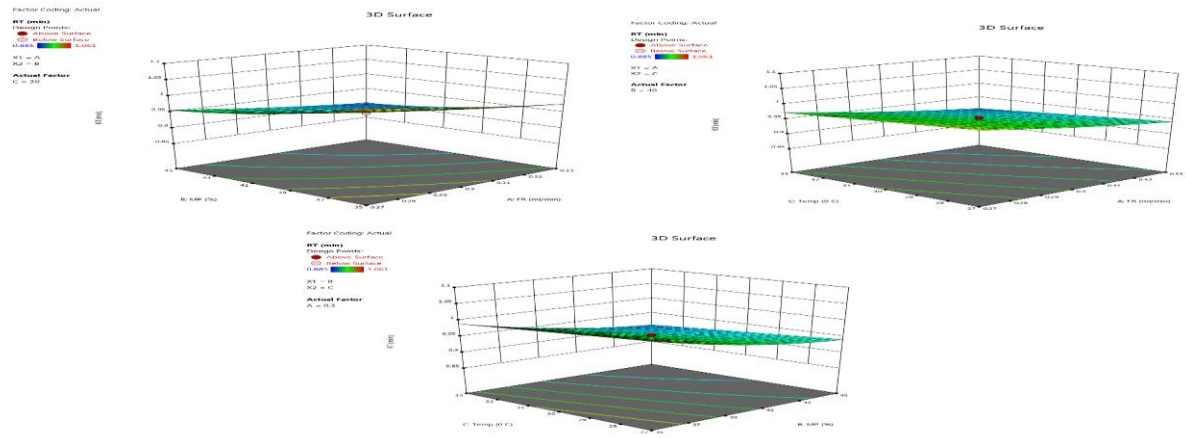
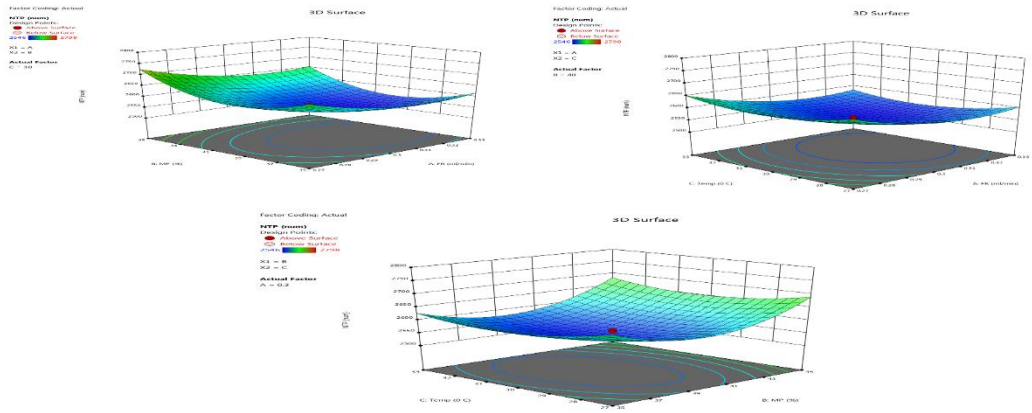


Figure4 2D Counter Plate of effect of column temperature and Mobile phase organic % Composition on the Number of theoretical Plates



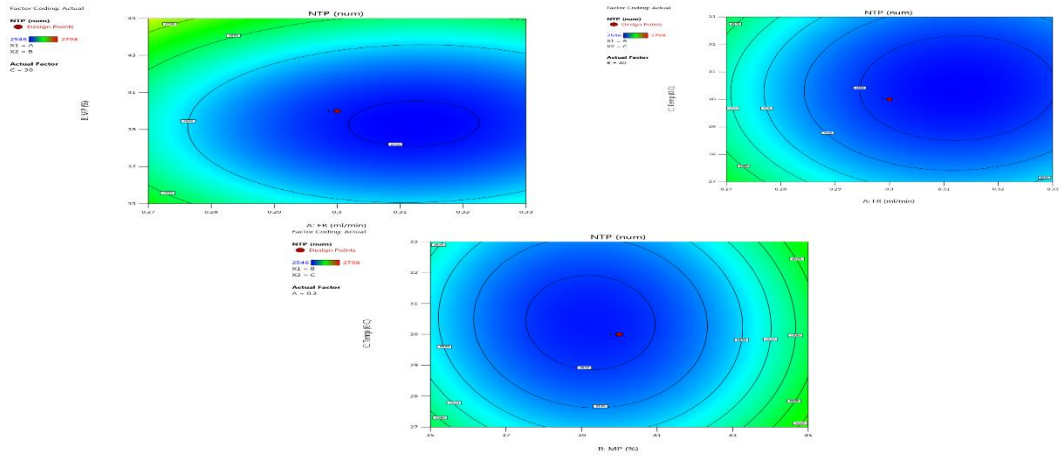


Figure5 3D Counter Plate of effect of column temperature and Mobile phase organic % Composition on the Number of theoretical Plates

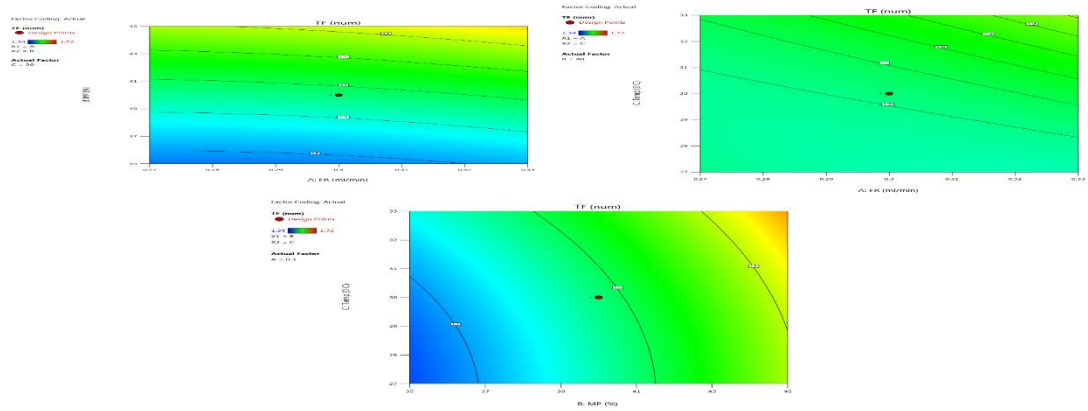


Figure6 2D Counter Plate of effect of column temperature and Mobile phase organic % Composition on the Tailing Factor

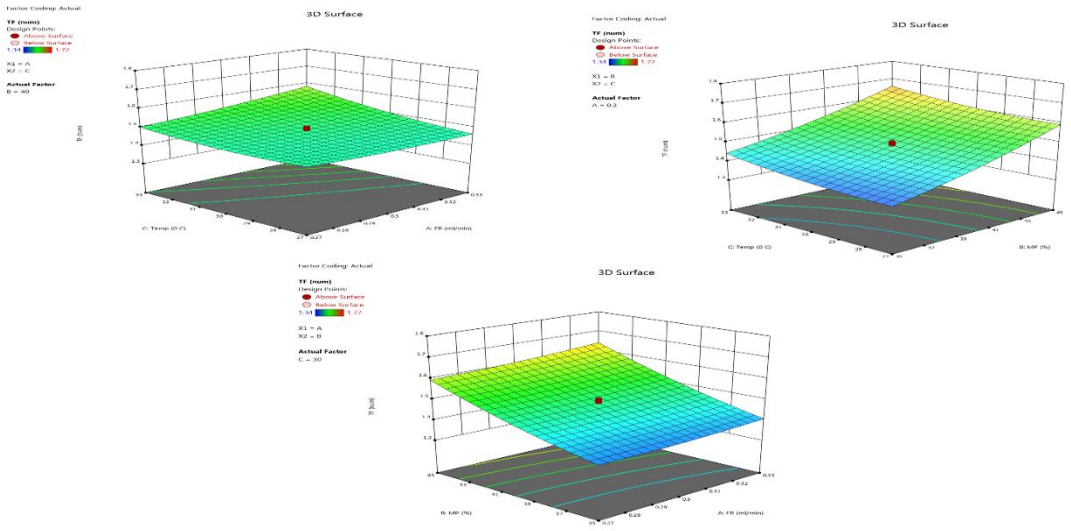


Figure7 3D Counter Plate of effect of column temperature and Mobile phase organic % Composition on the Tailing Factor

Factors

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	FR	0.2700	0.2700	0.3300	0.0000	Actual
B	MP	35.00	35.00	45.00	0.0000	Actual

C	Temp	27.00	27.00	33.00	0.0000	Actual

Point Prediction

Two-sided Confidence = 95% Population = 99%

Solution 1 of 100 Response	Predicted Mean	Predicted Median	Observed	Std Dev	SE Mean	95% C low fo Mean	95% C high fo Mean	95% T low fo 99% Pop	95% T high fo 99% Pop
RT	1.0583	1.0583		0.005986	0.004899	1.0473	1.0692	1.0248	1.0917
NTP	2715.89	2715.89		9.20746	7.53533	2699.1	2732.6	2664.4	2767.3
TF	1.38833	1.38833		0.0121063	0.00990776	1.36626	1.41041	1.3207	1.45597

Confirmation

Two-sided Confidence = 95%

Solution 2 o 100 Response	Predicted Mean	Predicted Median	Observed	Std Dev	n	SE Pred	95% P low	Data Mean	95% P high
RT	1.0583	1.0583		0.00598672	1	0.00773601	1.04106		1.07553
NTP	2715.89	2715.89		9.20746	1	11.8978	2689.38		2742.4
TF	1.38833	1.38833		0.0121063	1	0.0156438	1.35348		1.42319

Factors

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	FR	0.2700	0.2700	0.3300	0.0000	Actual
B	MP	35.00	35.00	45.00	0.0000	Actual
C	Temp	27.00	27.00	33.00	0.0000	Actual

	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
RT	0.952163	**-0.0310898**	**-0.0404024**	**-0.0159094**	0.0025	-0.001	-0.00025	**0.00435396**	**0.0131928**	-6.54583E-05
p-value		**< 0.0001**	**< 0.0001**	**< 0.0001**	0.2649	0.6467	0.9083	**0.0201**	**< 0.0001**	0.9677
NTP	2553.39	**-31.5823**	**22.3463**	**-8.12775**	-4.25	-2.75	4.5	**38.1328**	**75.4327**	**34.0669**
p-value		**< 0.0001**	**< 0.0001**	**0.0085**	0.2209	0.4180	0.1970	**< 0.0001**	**< 0.0001**	**< 0.0001**
TF	1.48509	**0.0166748**	**0.1105**	**0.0355463**	0.00625	**0.02375**	-0.00125	0.00651378	**0.0153526**	**0.0153526**
p-value		**0.0005**	**< 0.0001**	**< 0.0001**	0.1749	**0.0002**	0.7762	0.0684	**0.0007**	**0.0007**

Optimized Chromatographic Conditions:

Column : ACQUITY UPLC HSS C18 Column, 130A, 1.7 um, 2.1 mm X 100 mm,
Mobile phase : 0.1% OPA: Acetonitrile (60:40)
Flow rate : 0.30 ml/min

Detector : PDA 215.0 nm
Temperature : 30.74^{0}C
Injection Volume : 1.0µL

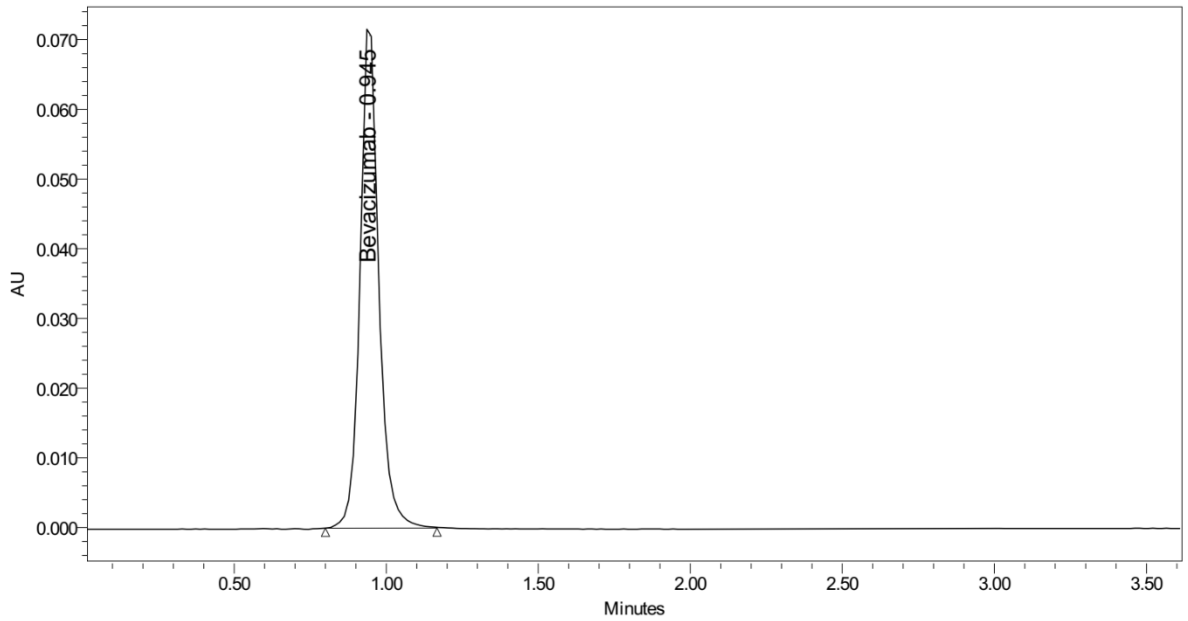


Figure 8. Chromatogram of final optimized method

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table: 6 System suitability parameters for Bevacizumab

S no	Bevacizumab		
Inj	RT(min)	USP Plate Count	Tailing
1	0.940	2694	1.16
2	0.940	2701	1.16
3	0.940	2694	1.15
4	0.944	2701	1.12
5	0.954	2710	1.17
6	0.955	2724	1.15

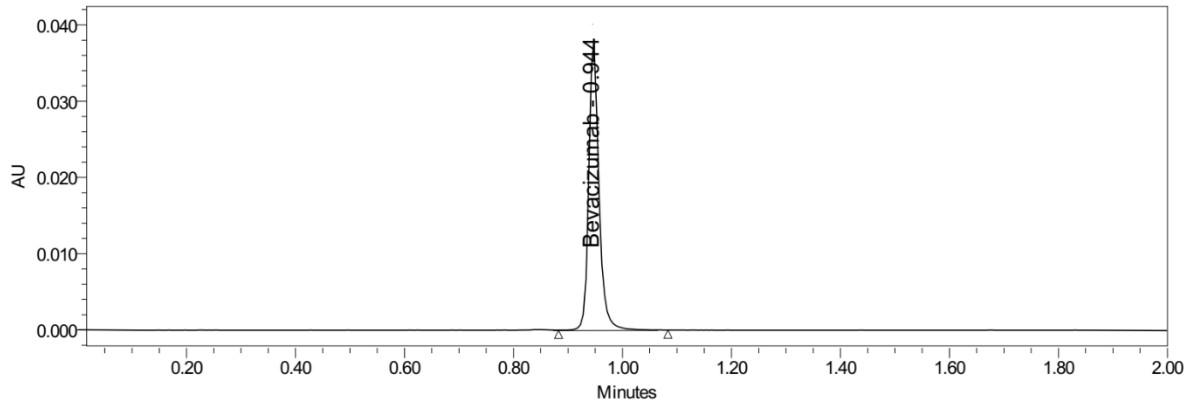


Figure 9 System suitability Chromatogram

Discussion: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Validation:

Specificity:

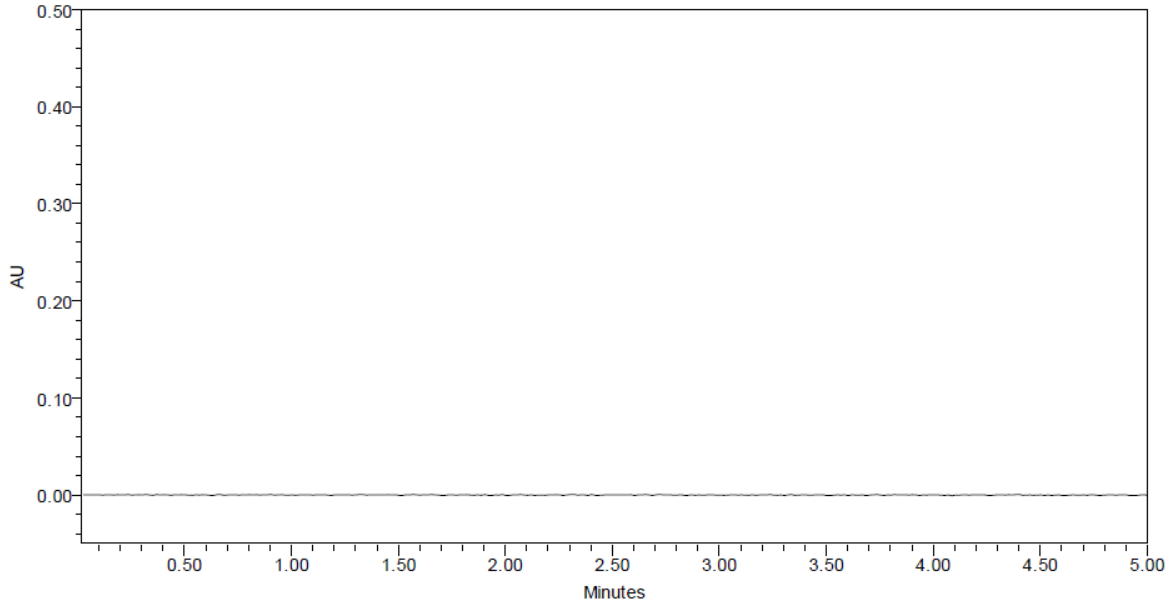


Figure10 Chromatogram of blank.

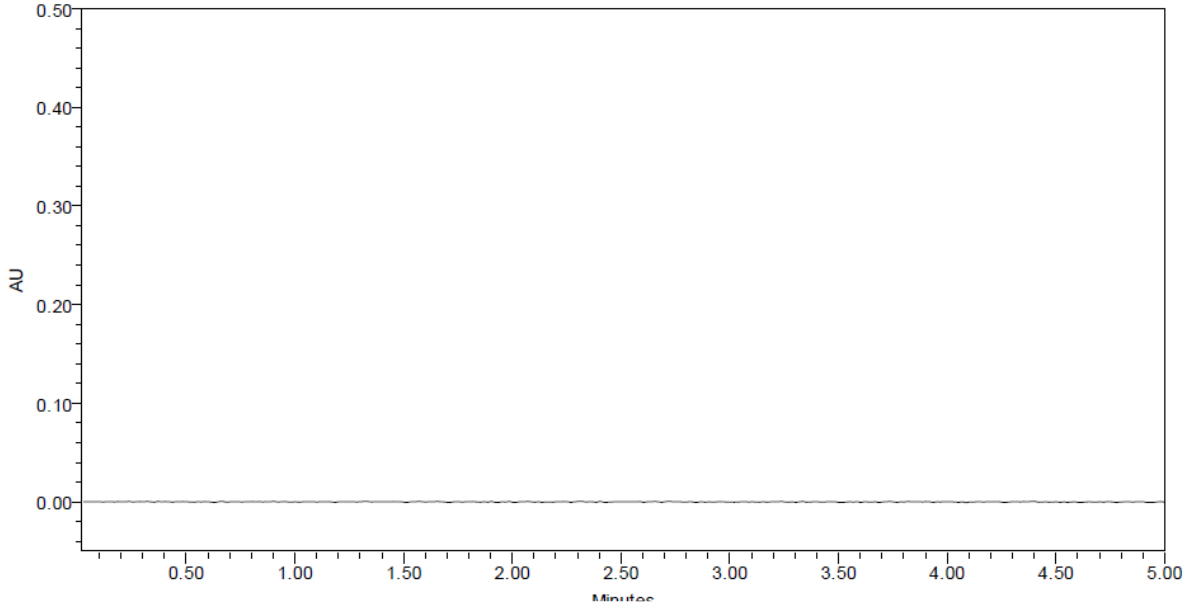


Figure11 Chromatogram of placebo

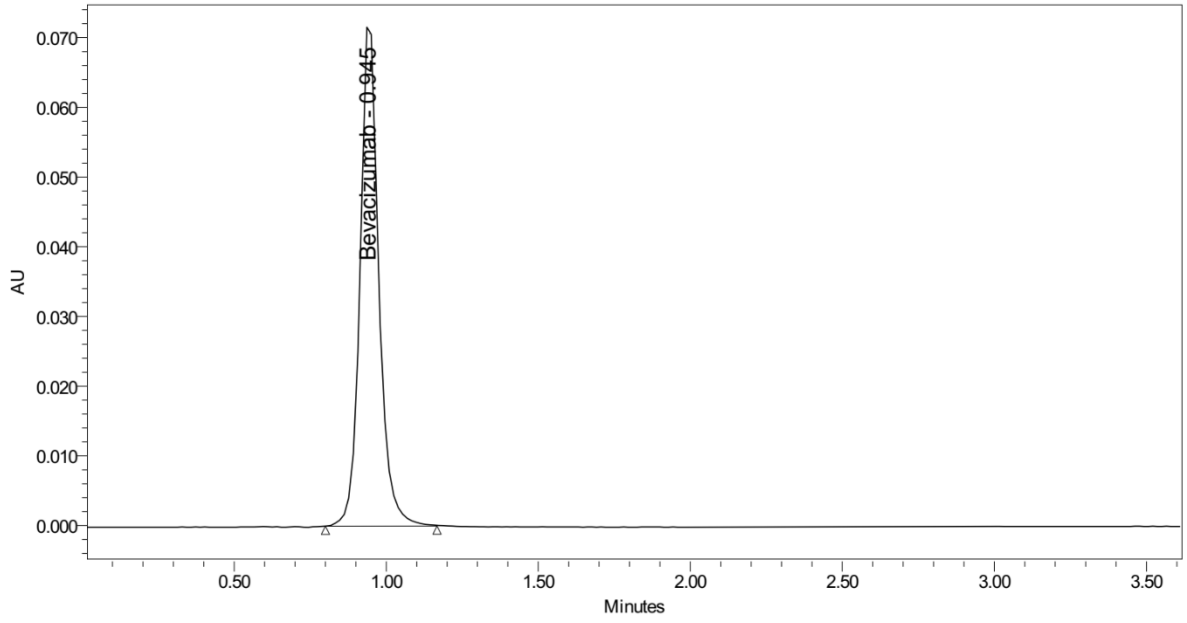


Figure 12 Optimized chromatogram

Discussion: Retention times of Bevacizumab were 1.043 min. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity:

Table 7 Linearity table for Bevacizumab.

Bevacizumab	
Conc (µg/mL)	Peak area
0	0
6.25	73271
12.5	145807
18.75	219397
25	293638
31.25	363514
37.5	430932

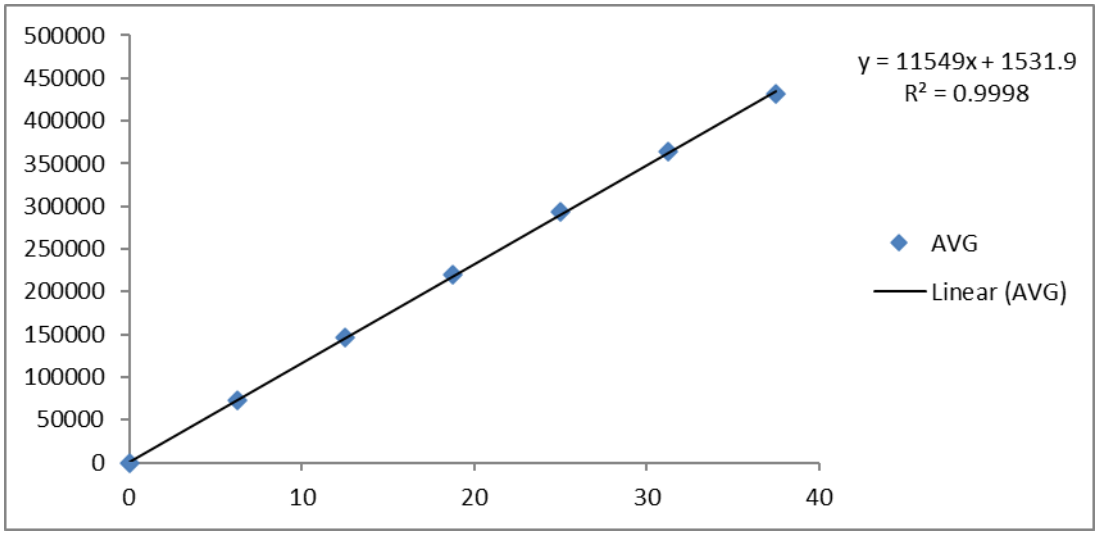


Figure13 Calibration curve of Bevacizumab

Discussion: Six linear concentration of Bevacizumab (6.25-37.5 µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Bevacizumab was y = 11549x + 1531.9. Correlation coefficient obtained was 0.999 for the two drugs.

Precision:

System Precision:

Table 8 System precision table of Bevacizumab

S. No	Area of Bevacizumab
1.	291055
2.	292147
3.	293642
4.	292725
5.	297759
6.	291210
Mean	293090
S.D	2482.0

%RSD	0.8

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. % RSD obtained as 0.8% for Bevacizumab. As the limit of Precision was less than "2" the system precision was passed in this method.

Repeatability:

Table 9 Repeatability table of Bevacizumab

S. No	Area of Bevacizumab
1.	293528
2.	292077
3.	293081
4.	293375
5.	292780
6.	293436
Mean	293046
S.D	548.4
%RSD	0.2

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated and obtained as 0.2% for Bevacizumab. As the limit of Precision was less than "2" the system precision was passed in this method.

Intermediate precision (Day _Day Precision):

Table 10 Intermediate precision table of Bevacizumab

S. No	Area of Bevacizumab
1.	289036
2.	291291
3.	289230
4.	287112
5.	291341
6.	289304
Mean	289552
S.D	1588.1
%RSD	0.5

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated and obtained as 0.5% for Bevacizumab. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Table 11 Accuracy table of Bevacizumab

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	12.5	12.447	99.58	99.75%
	12.5	12.506	100.04	
	12.5	12.478	99.82	
100%	25	24.958	99.83	
	25	24.885	99.54	
	25	24.815	99.26	
150%	37.5	37.43	99.82	
	37.5	37.53	100.07	
	37.5	37.42	99.78	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.75% for Bevacizumab.

Sensitivity:

Table 12 Sensitivity table of Bevacizumab

Molecule	LOD	LOQ
Bevacizumab	0.05	0.16

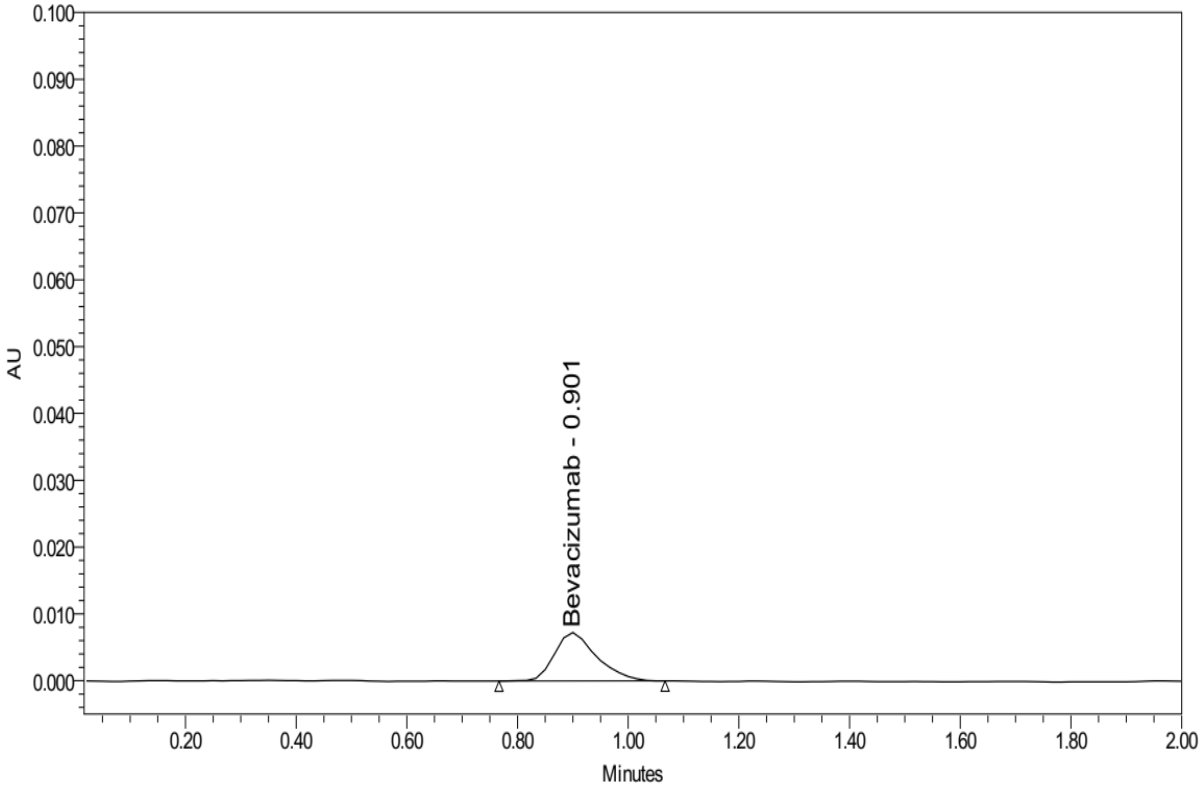


Figure 14 LOD Chromatogram of Standard

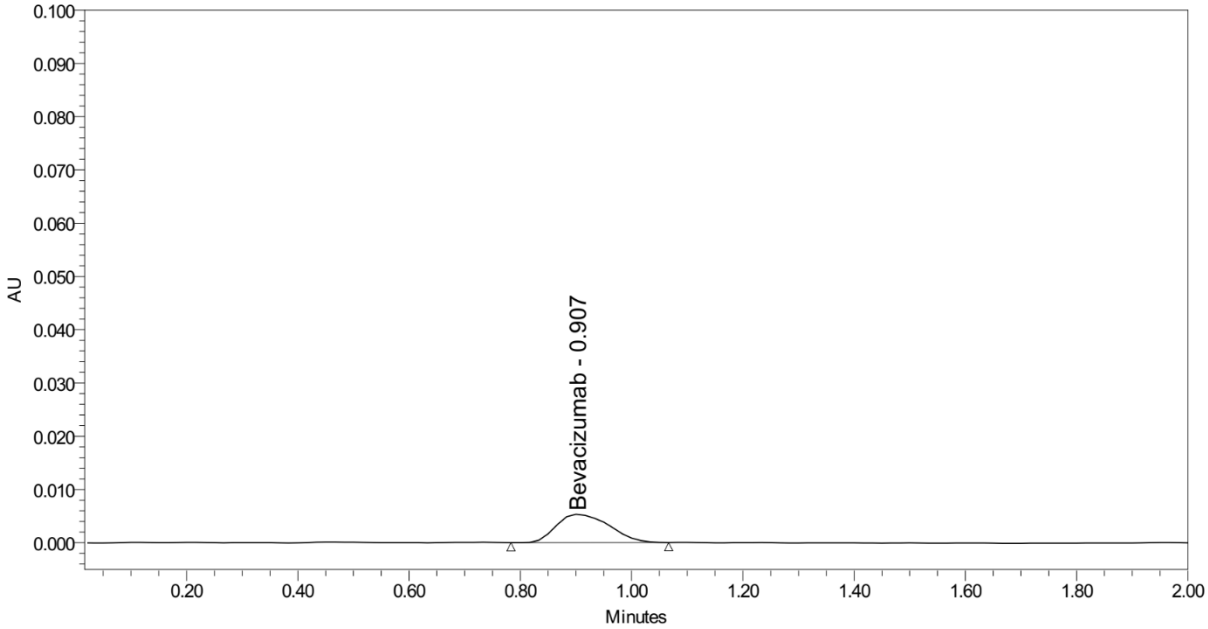


Figure 15 LOQ Chromatogram of of Standard

Robustness:

Table 13 Robustness data for Bevacizumab

S.no	Condition	%RSD of Bevacizumab
1	Flow rate (-) 0.1ml/min	0.8
2	Flow rate (+) 0.3ml/min	0.9
3	Mobile phase (-) 35B:65A	0.4
4	Mobile phase (+) 45B:55A	0.9
5	Temperature (-) 24°C	0.8
6	Temperature (+) 35°C	0.8

Discussion: Robustness conditions like Flow minus (0.1ml/min), Flow plus (0.3ml/min), mobile phase minus (35B:65A), mobile phase plus (45B:55A), temperature minus (24°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay: Avastin bearing the label claim Bevacizumab 25mg/4ml. Assay was performed with the above formulation. Average % Assay for Bevacizumab obtained was 99.79%.

Table 14 Assay Data of Bevacizumab

S.no	Standard Area	Sample area	% Assay
1	291055	293528	99.95
2	292147	292077	99.46
3	293642	293081	99.80
4	292725	293375	99.90
5	297759	292780	99.69
6	291210	293436	99.92
Avg	293090	293046	99.79
Stdev	2482.0	548.4	0.19
%RSD	0.8	0.2	0.2

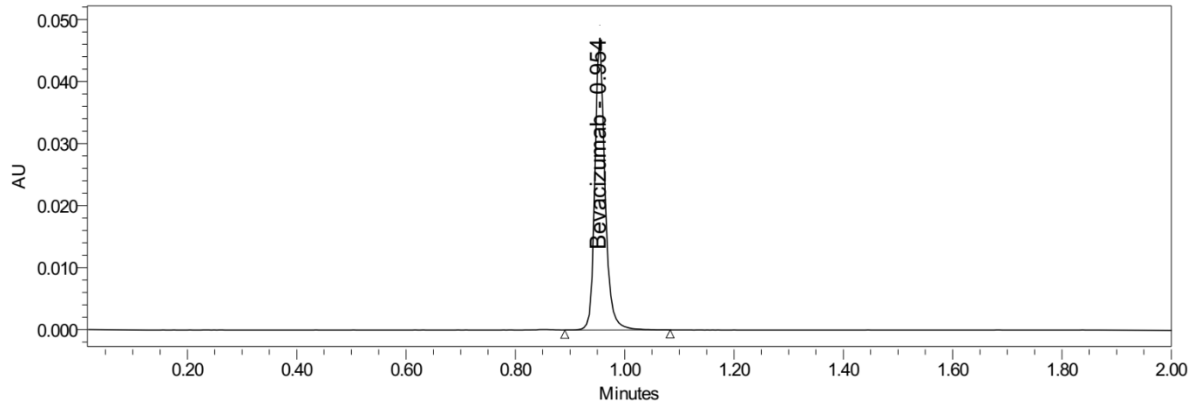


Figure 16 Chromatogram of working standard solution

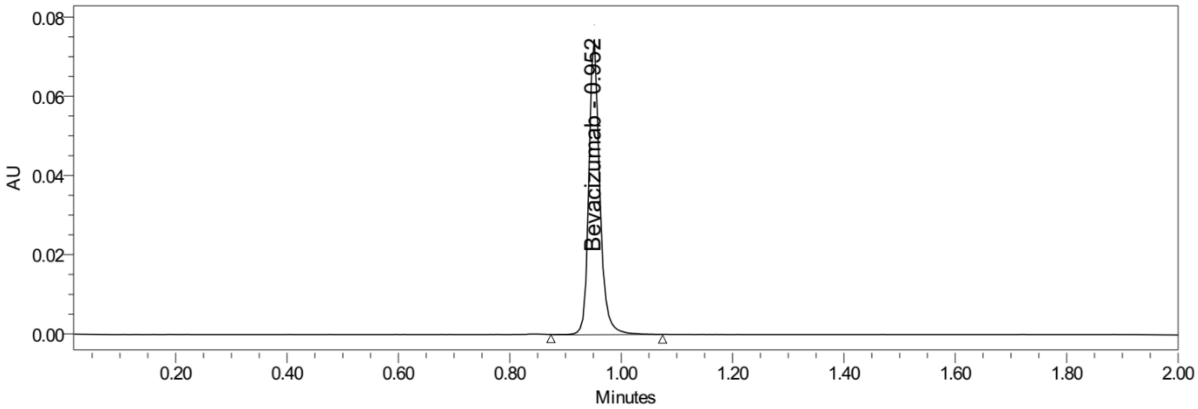


Figure 17 Chromatogram of working sample solution

Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation

Table 15 Degradation Data of Bevacizumab

S.NO	Degradation Condition	% Drug un Degraded	% Drug Degraded
1	Acid	95.62	4.38
2	Alkali	95.65	4.35
3	Oxidation	93.33	6.67
4	Thermal	97.60	2.40
5	UV	98.34	1.66
6	Water	99.32	0.68

Discussion: Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time.

SUMMARY

Table 16 Summary Table

Parameters	**Bevacizumab**	**LIMIT**
Linearity Range(µg/ml)	6.25-37.5 µg/ml	R< 1
Regression co-efficient	0.999	
Slope(m)	11549	

Intercept(c)		1531.9	
Regression equation (Y=mx+c)		y = 11549x + 1531.9	
Assay (% mean assay)		99.79%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		0.8	NMT 2.0%
Method precision %RSD		0.2	NMT 2.0%
Accuracy %recovery		99.75%	98-102%
LOD		0.05	NMT 3
LOQ		0.16	NMT 10
Robustness	**FM**	0.8	%RSD NMT 2.0
	FP	0.9	
	MM	0.4	
	MP	0.9	
	TM	0.8	
	TP	0.8	

CONCLUSION

A Simple, sensitive, specific, and precise RP-UPLC method for the Determination of Bevacizumab in API and pharmaceutical dosage form. Retention time of Bevacizumab was found to be 0.945 min. %RSD of the Bevacizumab were and found to be 0.8. %RSD of Method precision of Bevacizumab was found to be 0.2 %Recovery was obtained as 99.75% for Bevacizumab. LOD, LOQ values obtained from regression equation of Bevacizumab were 0.05, 0.16. Regression equation of Bevacizumab is y = 11549x + 1531.9. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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