EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 8
337-344
Year: 2019

HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF AXITINIB IN TABLET DOSAGE FORM

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Article Received on 07/06/2019 **Article Revised on 27/06/2019** **Article Accepted on 17/07/2019**

ABSTRACT
A simple, accurate, rapid and precise isocratic reverse phase high performance liquid chromatographic method has been developed and validated for the determination of Axitinib in tablet dosage form. The chromatographic separation was carried out with a discovery analytical column (250×4.6mm, 5µm), a mixture of 0.1% ortho phosphoric acid: acetonitrile in the ratio of 55:45 as mobile phase, at a flow rate of 1.0 ml/minute maintaining the temperature at 30ºc. UV detection was performed at 322 nm. The retention times were 2.537for Axitinib. The method was validated according to ICH guidelines and the acceptance criteria of results for accuracy, precision, linearity, robustness, limit of detection, limit of quantification and ruggedness were met in all cases. The % RSD values for Axitinib in precision study was found to be 0.41%. The linearity of the calibration curve for analyte in the desired concentration range was good (r^2>0.999). The high recovery and value of low relative standard deviation confirm the suitability of the method for routine evaluation of Axitinib in pharmaceutical dosage forms.

KEYWORDS: Axitinib, HPLC, Method development, validation.

INTRODUCTION
Axitinib (AXB), a research product of a reputed phamaceutical organization named Pfizer, is a recommended drug for renal cell carcinoma.[1-4] It acts by inhibiting small molecule tyrosine kinase into the cell. Though there are certain reports available on fatal adverse effects, AXB has shown a substantial increase in survival without further progression. AXB is officially approved by the U.S. Food and Drug Administration. Chemically the compound is N-Methyl-2-[[3-[(1E)-2-(2-pyridinyl) ethenyl]-1H-indazol-6-yl]thio]benzamide. Figure 1 represents the structure of AXB.

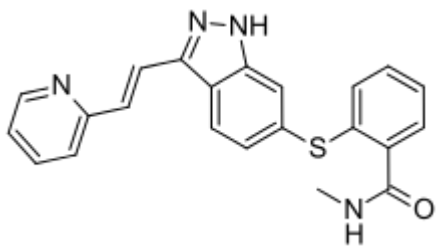

Figure 1: Chemical structure of Axitinib.

Literature survey[5-8] helps us to get motivated and go for the present research work. There are certain assay methods available for this compound. Albiol-Chiva, Esteve-Romero and Peris-Vicente developed a method to determine AXB, lapatinib and afatinib in plasma by micellar liquid chromatography. Achanta Suneetha, Sharmila Donepudi developed HPLC method for the estimation of AXB in rabbit plasma. Yuanheng MaJ, SuShow, Tianyan Zhou developed a liquid chromatography-tandem mass spectrometric technique for the determination of this compound in nude mouse plasma. Ranjeetha M, Niranjan M S, Chaluvaraju K C, Mamatha M, Darshitha R and Varsha developed derivative Spectroscopic method for the determination of AXB. But the extensive use of this compound makes a scope to work further so that a more simple method will be available for the regular estimation purpose.

MATERIALS AND METHODS
Instruments: HPLC make-waters 2690 with detector PDA-2996. Column as ODS C18 (250mm x 4.6 mm, 5m). Balance analytical- ER-180A, Sartorius

Microbalance-M500P, Thermo scientific pH Meter, Sar torius Sonicator, Empower V 1.2.2.1 Software.

Chemicals: Ortho phosphoric acid (AR) make- SDF, HPLC grade Water, HPLC grade acetonitrile. All these three solvents are products of Merck.

Preparation of mobile phase: 0.1% OPA and acetonitrile was taken in the ratio 55:45. This solution was filtered through a 0.45 µ membrane filter under vacuum, degassed in ultrasonic water bath for around 5 minutes.

Preparation of working stock solution (50µg/ml): 5 mg of SBR was weighed and finely powdered and transferred into 10 ml volumetric flask, diluted up to the mark with 7 ml mobile phase, sonicated for 30 minutes and made up the final volume with mobile phase.

Preparation of working standard solution: From the above stock solution, 1 ml was pipeted out in to a 10ml volumetric flask and then made up to the final volume with mobile phase to get the concentration of 50 µg/ml AXB and considered it as a standard 100%. This solution was filtered through 0.45 µm membrane filter.

Label Claim: 5 mg of Axitinb.

Method development

To develop a new method[9,10] for estimation work several trials were conducted so that we can achieve most suitable chromatographic condition and the best results. The initial attempt was to use as much low part of organic solvents for the purpose of elution. But increased part of aqueous solvents in our mobile phase resulted in extending of retention time for all the compounds. But reasonable retention time, value of tailing factors, number of theoretical plates and all were found to be within the validation limit while using optimized chromatographic condition.

Method validation

The method was evaluated[11-13] as per protocol designed by ICH.[15] The evaluation parameters took into consideration were system suitability parameters, precision accuracy, intermediate precision, linearity, limit of quantification, limit of detection, robustness studies etc.

System suitability parameters: For one analytical method validation system suitability parameters to be determined by preparing standard solutions of the compounds of specific concentration and the solutions to be injected six times and the parameters like peak tailing, theoretical plate count, retention time etc to be determined.

Specificity: Checking of interference if any in the optimized method. We should not find any interfering peak in blank in this method so that the method can be considered as specific.

Accuracy: The accuracy for a developed HPLC method is to be examined by calculating the extant of recoveries of all the compounds by a procedure called standard addition. Correct amount of drug solutions (standard) of that particular project (each drug 50%, 100%, and 150%) to be added and injected to pre-quantified solution of sample. The quantity of each substance recovered to be determined.

Precision: The experimental repeatability as well as intermediate precision to be examined by repeatedly applying six injections containing the compounds with specific concentration at two subsequent days. Number of theoretical plates, retention time, peaks resolution, peak symmetry etc must be the subject of observation.

Linearity: A series of gradually increased concentration for the entire range of compounds to be designed to conduct linearity test. To build up calibration curve, concentration and area should be considered at X and Y axis respectively.

LOD and LOQ: Calculation for Limit of detection as well as Limit of quantification to be been done by using standard Equations. LOD = $3.3\times\sigma/S$, LOQ = $10\times\sigma/S$. Here σ denotes for standard deviation of intercepts of regression lines, S denotes for slope.

Robustness: Evaluation for robustness to be conducted by making alteration in different chromatographic parameters. These parameters included flow rate, temperature, mobile phase composition etc.

Assay of marketed formulation: Assay of marketed product must be carried by injecting sample corresponding to equivalent weight into HPLC system, percentage purity to be found out by the following formula,

$$Conc_{unknown} = \left(\frac{Area_{InternalStd.in\ known}}{Area_{InternalStd.in\ unknown}}\right) \times \left(\frac{Area_{unknown}}{Area_{known}}\right) \times (Conc_{known})$$

Stability Studies: Force degradation study for the compound was conducted by providing different physical and chemical environment. The procedure for all these stability studies is described below.

Oxidative Degradation Studies: To 1 ml of stock solution of, 1 ml of 20% hydrogen peroxide (H_2O_2) was added. The obtained solution was kept for 30 min at around 60ºc. This resultant solution was diluted to obtain 100µg/ml and 10 µl of the solution was injected into the system. The chromatograms were recorded to assess the stability of the sample.

Acid Degradation Studies: To 1 ml of stock solution of 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at around 60ºc. This resultant solution was

diluted to obtain 100µg/ml solution and 10 µl solution was injected into the system. The chromatograms were recorded to assess the stability of sample in acidic environment.

Alkali Degradation Studies: To 1 ml of stock solution of, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at around 60ºc. This resultant solution was diluted to obtain 100µg/ml and 10 µl was injected into the system. 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 around 6 hours to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100µg/ml and10µl were injected into the system. The chromatograms were recorded to assess the stability of the sample.

Photo (UV) Stability Studies: The photochemical stability of the drug was studied by exposing 100µg/ml solution to UV Light by keeping the beaker in UV Chamber for around 7days. For HPLC study10 µl was injected into the system. The chromatograms were recorded to assess the stability of sample.

Neutral (water) Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for around 6hours at a temperature of 60ºc. For HPLC study, the resultant solution was diluted to100 µg/ml solution. 10 µl was injected into the system. The chromatograms were recorded to assess the stability of the sample.

RESULTS AND DISCUSSION

Method development: A unique method of assay was innovated by using columns of different length and make. Mobile phases containing various compositions with different proportions were tried by taking standard as well as sample in individual. Column or oven temperature, flow rate, different buffers (salt and acid combination) with slightly varying pH value and solvents were applied. Whichever the different mobile phases were prepared, subjected for filtration through membrane filters prior of their use. The mobile phase containing a mixture of 0.1% OPA: Acetonitrile in the ratio of 55:45 was considered as the best to obtain peaks of AXB at 2.537 minutes.

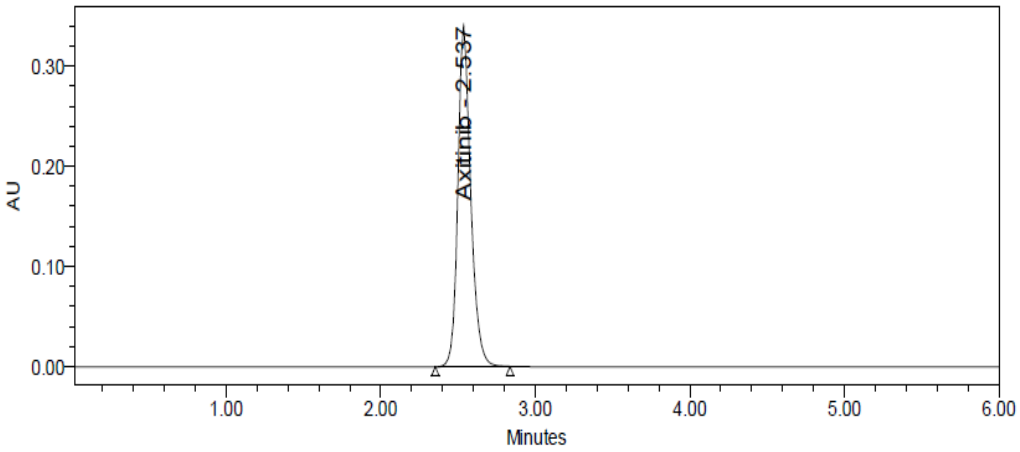

Figure 2: Optimized chromatogram of Axitinib.

Validation Results

Results of system suitability: The optimized chromatographic procedure as developed resulted in the elution of AXB at 2.537 minutes. Figure 2 is the representative chromatogram of standard solution of AXB. System suitability results were evaluated by taking six replicates of standard solution at 50µg/ml for the compound as mentioned respectively. Table 1 narrates about the results of system suitability parameters.

Table 1: Results of system suitability parameters.

Compound	Rt(Minutes)	Area	USPplate count	Tailing factor
Axitinib	2.537	1960954	4524	1.21

Results of accuracy studies: Accuracy of the method was well established from the results of percentage recovery. It was calculated from the amount of compounds recovered by comparing the peak average areas observed for standard and sample solutions. The percentage was found in the range of 98.50 – 101.90% for SBR as given in table 2.

Table 2: Results of Accuracy studies.

% Lavel	Amount spiked	Amount recovered	% Recovery	Mean recovery
50%	50	49.20	98.40	99.18%
	50	49.93	99.86	
	50	49.16	98.32	
100%	100	99.14	99.14	
	100	99.11	99.11	
	100	100.22	100.22	
150%	150	148.11	98.74	
	150	150.01	100.00	
	150	148.28	98.85	

N = 3 for each spiked standard.

Results of precision studies: The repeatability (intra-day trials) and intermediate precision (inter-day trials) studies for AXB revealed slight variations in the repetitive trial values (% RSD < 1.5) as narrated in table 3 indicating actual precision of the method.

Table 3: Results of precision studies.

S.No	Peak area of AXB standard (intraday)	Peak area of AXB standard (interday)
1	1947614	2021631
2	1937032	2020981
3	1949310	2043043
4	1929295	2034442
5	1942237	2048017
6	1946735	2043331
Mean	1942037	2035241
SD	7661.8	11651.3
%RSD	0.41	0.60

N = 6

Linearity and regression analysis: Concentration range of 25 µg/ml -150 µg/ml for AXB was designed for linearity test. Table 4 explains about appropriateness of the developed method. Sensitivity of the new method was good enough. With very low concentration the response in graph was sufficient to read and calculate all the results of regression analysis. Results of linearity test revealed that the Y intercept value was19669, value of correlation coefficient and slope value 39634, LOD 0.295µg/ml and LOQ 0.895µg/ml for AXB was respectively.

Table 4: Results

Linearity Level (%)	Concentration (ppm)	Area
0	0	0
25	25	572954
50	50	1041068
75	75	1485761
100	100	2032914
125	125	2490224
150	150	2980556

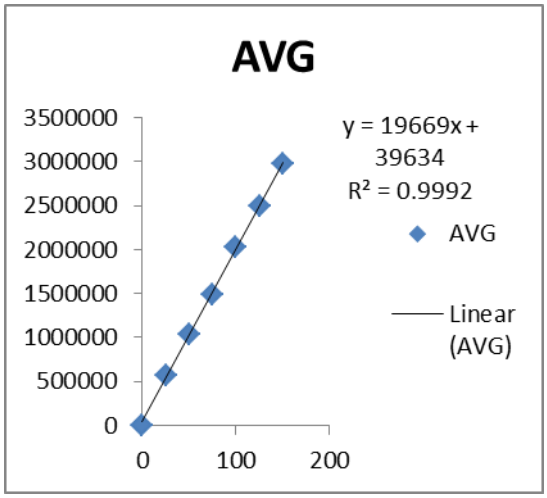

Figure 3: Linearity Plot.

Table 5: Sensitivity and regression analysis.

Parameters	Axitinib
Linearity (µgm/ml)	25 µg/ml- 150 µg/ml
Correlation Coefficient.(r)	0.999
Regression slope	19669
SD of Slope	0.39
Regression Intercept (mean)	39634
%RSD of Intercept	0.28
LOD	0.06µg/ml
LOQ	0.18µg/ml

Results of robustness studies: This exercise had been done by bringing marginal variation in certain chromatographic parameters namely increasing and reducing flow rate, variation in the ratio or proportion of aqueous phase and organic one, temperature status of column etc. Retention time, plate counts as well as asymmetric or tailing factor etc was obtained with very marginal variation. All the observed analytical values are given in table 5 as tabular form.

Table 6: Results of robustness studies.

	Chromatographic condition	Retention time	USP plate count	Tailing factor	% Assay
Axitinib	Flowrate1.2ml/min	2.501	4422	1.21	99.03
	Flowrate0.8ml/min	2.593	4608	1.21	99.87
	Buffer 60 parts	2.577	4583	1.21	98.98
	Buffer 50 parts	2.519	4517	1.21	99.40
	Temperature(35°c)	2.533	4515	1.21	100.03
	Temperature(25°c)	2.536	4688	1.21	98.86
	Mean	2.543	4555	1.21	99.36

N = 3

Assay of marketed formulation: The formulation (Tablet- Inlyta-5) was procured from Medindia Pharma network. Ten tablets had been chosen, weighed and collected in a clean and dry mortar. Tablets were triturated into powder form and then collected an equivalent quantity of 10mg of AXB in a dry volumetric flask (100 ml). Entire quantity of powder was treated with diluent and then subjected for sonication. The volume was made with diluent. 1 ml of the solution was pipetted out into a volumetric flask (10 ml) and the volume was made with diluent. 10 µl of resultant solution was injected to the Chromatographic system and analytical result was studied as compared to that of standard preparation. Peak area response was taken into consideration. Mean assay value for six sample trial was found to be 99.94%.

Results of degradation studies: Stability studies for AXB were conducted in different physicochemical conditions namely acid, alkali, peroxide, UV radiation, higher temperature and neutral conditions. Solutions of AXB shown that they were stable enough under almost all stress environment. Very minor degradation took place and was observed in oxidative and higher heat

conditions. Figure 4 to 9 represent chromatogram after all those different stress condition. Table 7 describes results of force degradation of AXB.

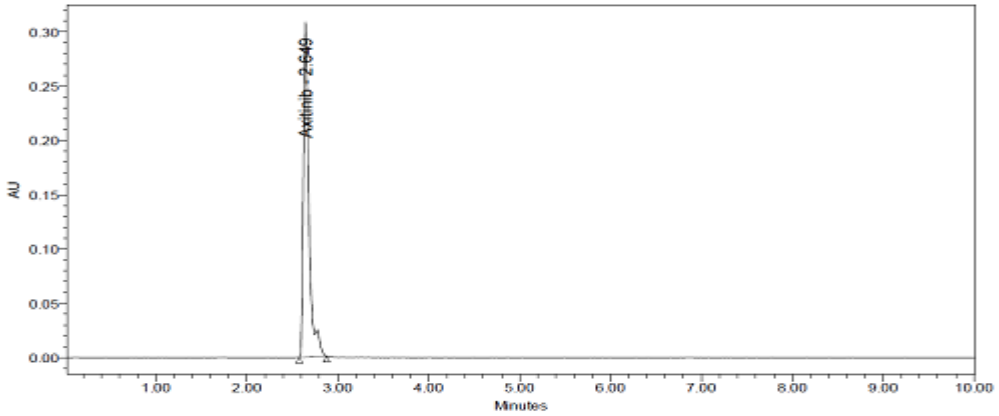

Figure 4: Chromatogram by degraded compound after acidic stress.

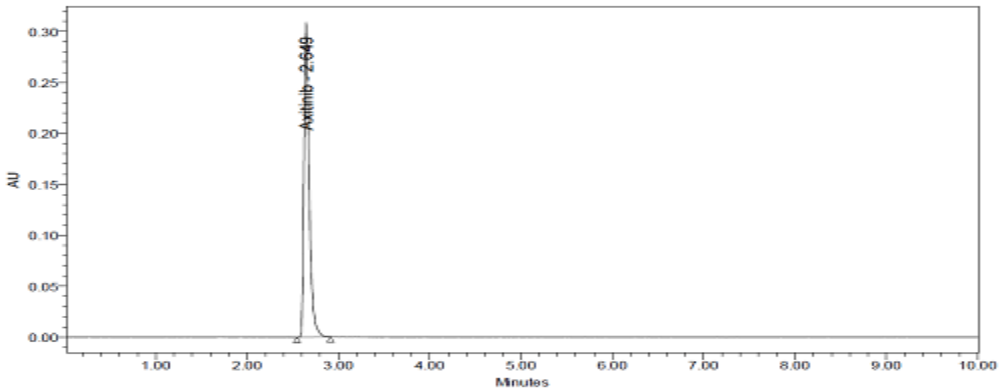

Figure 5: Chromatogram by degraded compound after alkaline stress.

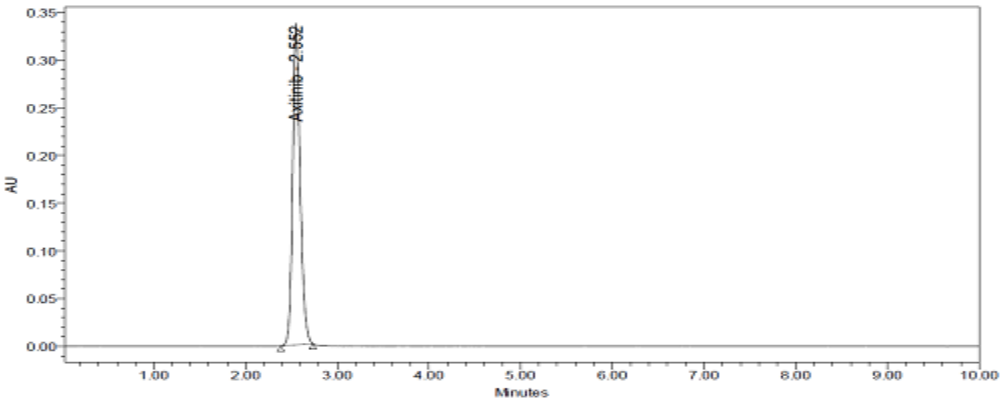

Figure 6: Chromatogram by degraded compound after oxidative stress.

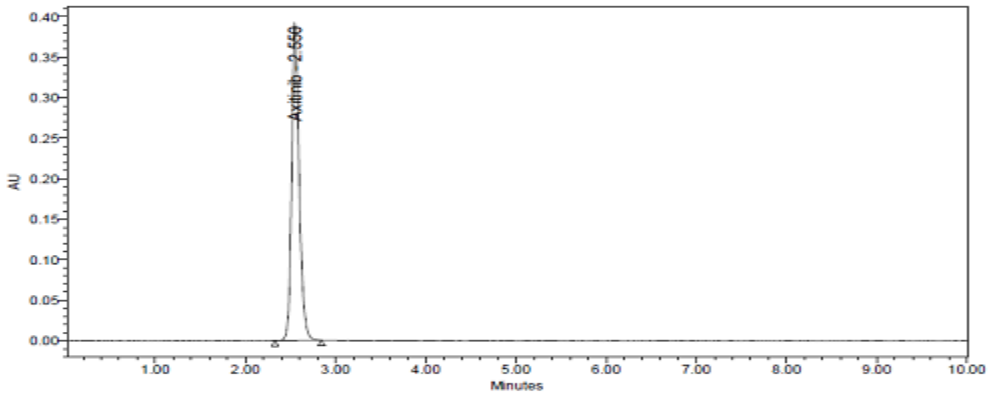

Figure 7: Chromatogram by degraded compound after thermal stress.

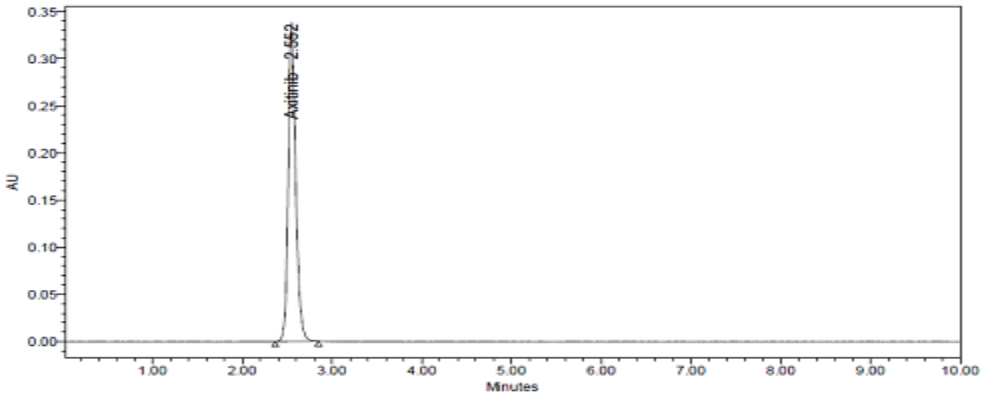

Figure 8: Chromatogram by degraded compound after UV radiation stress.

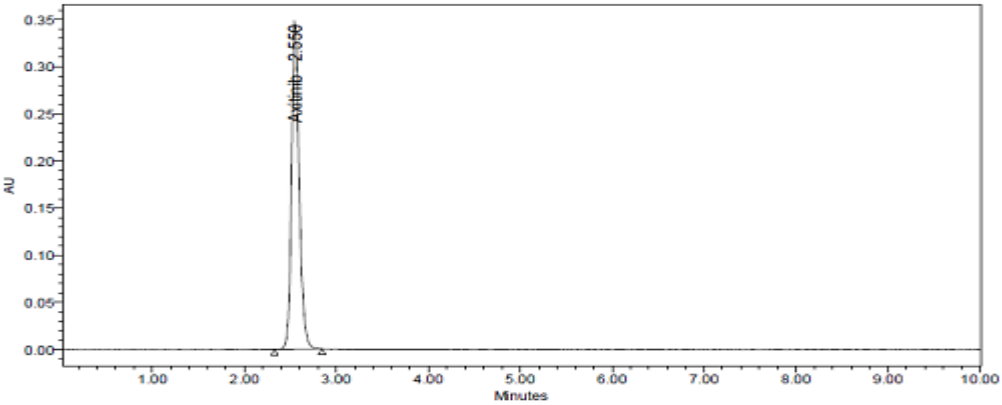

Figure 9: Chromatogram by degraded compound after neutral stress.

Table 7: Results of degradation studies.

Sl No.	Physico-chemical condition	% of degradation	Purity angle	Purity threshold
1	Acid	4.52	1.104	1.287
2	Alkali	2.50	2.063	3.302
3	Oxidative	1.43	0.071	0.274
4	Thermal	0.40	0.115	0.282
5	UV radiation	0.49	0.111	0.287
6	Water	0.71	0.179	0.284

CONCLUSION

The present HPLC method for the determination of Axitinib was found to be one of the least time consuming, simple, highly accurate technique as all the validation results of all parameters were with very low value of %RSD. At the same time it also proved that the innovated technique is a precise and robust method. Therefore the above narrated novel analytical technique

is a prefered and suitable one for evaluation of bulk and tablet formulation of the drug in laboratory on regular basis.

ACKNOWLEDGEMENTS

We are very much thankful to Dr. A. Srinivasa Rao, Professor and Principal Bhaskar Pharmacy College, Hyderabad for his guidance, kind help and constant encouragement at every step during the progress of this research work. We also express our gratitude to Dr. V.V. Rao, CEO, and Mr. J.V. Krishna Rao, secretary, J.B. Group of Educational Institution for providing a healthy working environment which is an essence in research field.

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