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METHOD DEVELOPMENT AND VALIDATION OF AFATINIB USING CHIRAL HPLC

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ABSTRACT

A sensitive and robust chiral high performance liquid chromatography (HPLC) with UV method was developed and validated for the quantification of R-isomer in afatinib as per ICH Q2 guideline. In this method, both R and S-isomers were well-separated on CHIRALPAK-IE column (250 X 4.6 mm X 5µ) in an isocratic elution mode with the mobile phase comprising of methanol: methyl tertiary butyl ether: diethyl amine (80:20:0.1; v/v) at a flow rate of 0.7 mL/ min. The column oven temperature was maintained at 20ºC and both afatinib and its enantiomer were monitored at 254 nm by UV detector. The validated method was found to be precise, accurate and linear from the range of LOQ level to 150% with respect to sample concentration and the correlation co-efficient was found to be 0.998. Limit of detection and limit of quantifications were found to be 0.00005 and 0.00015 mg/mL, respectively. The validated method was found to be very sensitive and the recoveries were found to be well within the range from 95.65% to 100.73% for R-isomer. Further, the solution stability was also established and the solutions were found to be stable upto 48hrs.

KEYWORDS: Afatinib, liquid chromatography, chiral analysis, method validation.

1. INTRODUCTION

Many of the new chemical entities under development in the pharmaceutical industry are chiral. The specific stereochemistry of these substances affects both the biological activity and commercial viability of the potential new drug. Thus, enantioselective separation techniques play a vital role in the development of these entities into commercial products.

Afatinib is a selective and irreversible inhibitor of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 and is used for the treatment patients with EGFR-mutant non-small cell lung cancer (Solca et al., 2012). It is an irreversible tyrosine kinase receptor inhibitor which was approved lately by USFDA for the treatment of metastatic non-small cell lung cancer (NSCLC).

Very few reports are present on determination of drug in bulk and dosage forms (Vejendla et al., 2015). Some stability indicating HPLC methods and forced degradation studies of AFT are available in the literature (Chavan et al., 2018; Kalariya et al., 2015; Devrukhakar et al., 2018; Chavan & Radhakrishnanand et al., 2018). Marwa et al., have used UPLC-DAD method for the development and validation followed by quantification of AFA in human plasma (Marwa et al., 2015). Two other HPLC methods have been reported on quantitation of Afatinib in tablets (Vejendla et al., 2015). Determination of Afatinib was reported in two recent studies in human plasma using UPLC-DAD (Marwa et al., 2015). Recently, several assays have been reported for the detection of afatinib in different biological matrices using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Hayashi et al., 2016; Kadi et al., 2016; Sparidans et al., 2016; Sparidans & Van Hoppe 2016; Stopfer et al., 2012).

To the best of our knowledge, there are no reports on the chiral method development and validation of Afatinib using HPLC-UV method. Hence, we aimed to develop and validate a simple, specific, rapid and sensitive and robust chiral HPLC method for the quantification of R-isomer in Afatinib.

2. Experimental

2.1. Chemicals and reagents

Afatinib (S-isomer) and its other isomer (R-) were obtained from local manufacturing unit located in Hyderabad, India. HPLC grade Methanol, Methyl Tertiary Butyl Ether (MTBE) procured from Merck, India and HPLC grade Diethyl amine procured from Sigma-Aldrich, Belgium.

2.2. Instrumentation, HPLC conditions and sample preparation

The HPLC analysis was performed using waters 2996 HPLC (Waters Corporation, USA) system with quaternary pump connected to degasser unit, Photo Diode array detector, column cooler and heater compartment and auto sampler with loop volume 50µL.

Afatinib and its R-isomer were separated on Daicel CHIRALPAK-IE column 250 X 4.6mm, 5µ with the mobile phase consisting of MeOH: MTBE: DEA (80:20:0.1). The mobile phase flow rate is 0.7 mL/min and the injection volume is 10µL. The column temperature is maintained at 20°C and the analysis is carried out at the detection wavelength of 254 nm. The sample concentration used was 0.5 mg/mL and separation of samples was achieved in an isocratic elution mode.

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic conditions

To obtain a sensitive and robust method, used several columns and made several trials to achieve the good separation of two isomers which has been mentioned in the succeeding text. First, used Chiral Pak ADH column with the mobile phase comprising of MeOH: MTBE: DEA (80:20:0.1) and the column temperature 20°C was used as starting conditions for method development. When these conditions are used, both the isomers were co-eluted (merged) as a single peak where peak shape was not symmetrical as shown in Figure 1. Secondly, mobile phase composition changed to MeOH: MTBE: DEA (85:15:0.1) even though could not see any separation. We have changed the column to CHIRALPAK-IE (250 X 4.6mm X 5µ) with the same mobile phase observed the separation but separation is not good enough as shown in Figure 2. Mobile phase composition changed to MeOH: MTBE: DEA (85:20:0.1) and checked the separation in the same column, found good separation as shown in Figure 3. To check further about the impact of column chemistry on separation, various immobilized chiral columns were screened, namely chiral Pak IA-3, chiral Pak IB-3, chiral Pak IC-3 and chiral Pak IG-3. With Chiral Pak IA-3, chiral Pak IB-3 chiral Pak IG-3, no separation observed and with chiral Pak IC-3 separation observed as shown in Figure 4, but the separation is not good as with the CHIRALPAK-IE. As a further trial to check impact of column temperature, increased the column temperature and observed that at higher temperature (30°C instead of 20°C) both the isomers were come closer and resolution gone bad as shown in Figure 5. Finally, CHIRALPAK-IE (250 X 4.6mm X 5µ) column and with the same mobile phase (MeOH: MTBE: DEA (80:20:0.1).) at reduced the column temperature (20°C) is a better method to get enough resolution with good peak shape. The UV wavelength was set at 254 nm and methanol was used as a diluent.

3.2. Method validation

The method was validated with respect to specificity, precision, accuracy, linearity, sensitivity, robustness and ruggedness/intermediate precision as per ICH Q2 guideline.

3.2.1. Specificity

The method specificity was assessed by comparing the sample chromatogram with blank chromatogram and racemic chromatogram. The blank solution was methanol which has been used as diluent (blank) to dissolve the drug. The data of specificity is provided in Table 1. From the chromatograms, it can be seen that the method is found to be specific. The representative chromatograms of blank, racemic mixture, afatinib sample and R-Isomer spiked sample are depicted in Figure 6, 7, 8 and 9.

3.2.2. Precision studies (Method precision and Intermediate Precision/Ruggedness)

Precision is the degree of reproducibility of an analytical method under normal conditions. The precision of the method was studied in terms of repeatability (intra-day precision) and intermediate precision (inter day precision) by preparing five individual sample solutions on different day, different analyst, different instrument and different column. The Standard deviation and %RSD calculated were provided in Tables 2 and 3. The data of precision studies indicate that the method is found to be precise and rugged. Precision study was also performed at LOQ level and %RSD was well within the acceptance criteria. The LOQ precision data are provided in Table 4.

3.2.3. Linearity and range

Linearity of an analytical method is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. Linearity was determined by a series of injections of five standards whose concentrations are ranging from LOQ (0.03%) to 200% of the specified concentration. The response was directly proportional to the concentrations of the analytes. A linear regression equation applied, and a good linearity was found, and the correlation co-efficient (R^2) was found to be greater than 0.998. The data of linearity are provided in Table 5 and calibration curve is provided in Figure 10.

3.2.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ values were found to be 0.00005 and 0.00015 mg/mL, respectively. The representative data are provided in Table 6 and chromatograms are provided in Figure 11 and 12.

3.2.5. Accuracy

Accuracy of the method was evaluated by standard addition method. The known amount of R-isomer spiked in the range of LOQ to 150% of specified level and the sample solutions were analyzed in triplicate as per

proposed method. The recovery was found to be well within the range from 95.65% to 100.73% for R-isomer and the representative data are provided in Table 7. Representative chromatograms are provided in Figure 13.

Table 1: Data of Specificity.

Injection	R-Isomer (Retention time)	S-Isomer (Retention time)
Blank	NA	NA
Racemic	11.589	12.562
Sample	11.616	12.748
R-isomer Spiked sample	11.585	12.748

Table 2: Data of Method Precision.

No. of Injections	Area of R-Isomer
Preparation-1	17245
Preparation-2	17425
Preparation-3	17358
Preparation-4	17752
Preparation-5	17546
Average	**17465**
STDV	**193.89**
%RSD	**1.11**

Table 3: Data of Intermediate Precision/Ruggedness.

No. of Injections(n=5)	Day-1 Analyst-1	Day-1 Analyst-2
	Area of R-Isomer	Area of R-Isomer
Injection-1	17258	17548
Injection-2	17664	17245
Injection-3	17542	17745
Injection-4	17645	17652
Injection-5	17542	17745
Average	**17530**	**17587**
STDV	**162.37**	**207.75**
%RSD	**0.93**	**1.18**
No. of Injections(n=5)	**Day-2 Analyst-1**	**Day-2 Analyst-2**
Injection-1	17568	17654
Injection-2	17363	17456
Injection-3	17645	17563
Injection-4	17756	17345
Injection-5	17562	17632
Average	**17579**	**17530**
STDV	**143.83**	**129.01**
%RSD	**0.82**	**0.74**
Analyst wise (n=10)	**Analyst-1 (Day-1&2)**	**Analyst-2 (Day-1&2)**
Average	**17555**	**17559**
STDV	**146.86**	**165.77**
%RSD	**0.84**	**0.94**
Days wise (n=10)	**Day-1 (Analyst-1&2)**	**Day-2 (Analyst-1&2)**
Average	**17559**	**17554**
STDV	**178.31**	**131.35**
%RSD	**1.02**	**0.75**

Table 4: Data of LOQ Precision.

No. of Injections	Area of R-Isomer
Injection-1	4945
Injection-2	4952
Injection-3	4951
Injection-4	4978
Injection-5	4911
Average	**4947**
STDV	**23.99**
%RSD	**0.48**

Table 5: Data of Linearity.

Con. (%)	Area of R-Isomer
0.03	4968
0.05	8347
0.1	18604
0.15	28521
0.2	35726
R^2	**0.998**

Table 6: Data of LOD and LOQ.

Parameter	Con.(%)	Con.(mg/mL)	RT	Area	USP S/N
LOD	0.01	0.00005	11.58	1888	29.61
LOQ	0.03	0.00015	11.58	4945	178.52

Table 7: Data of Recovery.

<table>
<tr><th>Accuracy
Con. (%)</th><th>Area of
R-Isomer + Sample</th><th>R-Isomer area
in Sample</th><th>R-Isomer area
in Standard</th></tr>
<tr><td>Accuracy at 0.05%</td><td>27796</td><td rowspan="4">19371</td><td rowspan="2">17617</td></tr>
<tr><td>Accuracy at 0.10%</td><td>37276</td></tr>
<tr><td>Accuracy at 0.15%</td><td>47496</td><td>R-Isomer area in LOQ Standard</td></tr>
<tr><td>Accuracy at 0.03% (LOQ)</td><td>24354</td><td>4947</td></tr>
<tr><td>% Recovery at 0.05</td><td colspan="3">95.65</td></tr>
<tr><td>% Recovery at 0.10</td><td colspan="3">101.63</td></tr>
<tr><td>% Recovery at 0.15</td><td colspan="3">106.43</td></tr>
<tr><td>% Recovery
at 0.03 (LOQ)</td><td colspan="3">100.73</td></tr>
</table>

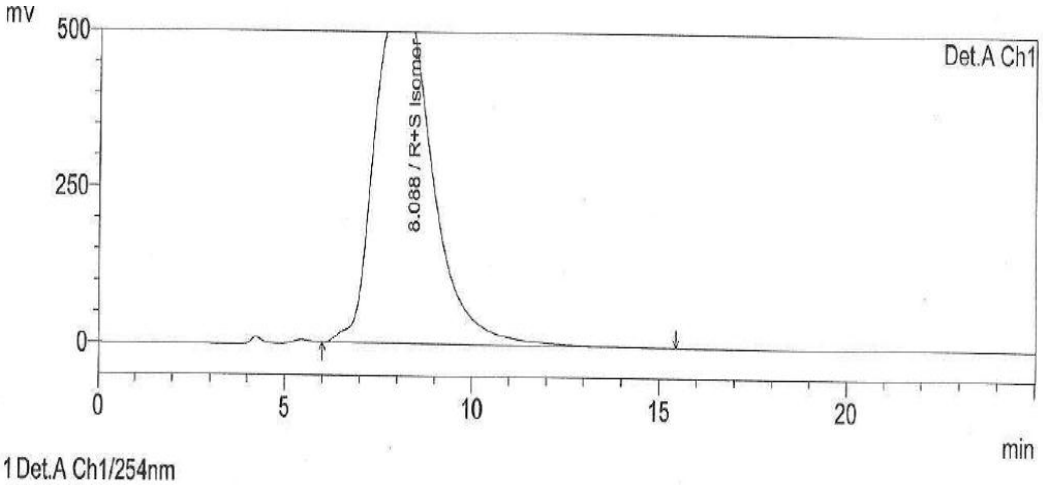
Table 8: Data of Robustness.

No. of Injections(n=5)	Flow rate at 0.6 mL	Flow rate at 0.8 mL
	Area of R-Isomer	Area of R-Isomer
Injection-1	18125	17852
Injection-2	18263	17456
Injection-3	18325	17693
Injection-4	18236	17963
Injection-5	18248	17423
Average	**18239**	**17677**
STDV	**72.56**	**237.71**
%RSD	**0.40**	**1.34**
No. of Injections (n=5)	**Column temp. at 15°C**	**Column temp. at 25°C**
Injection-1	16892	18123
Injection-2	16852	18243
Injection-3	16523	18235
Injection-4	16652	18325
Injection-5	16723	18452
Average	**16728**	**18276**

STDV	**150.08**	**122.02**
%RSD	**0.90**	**0.67**

Table 9: Data of Solution Stability.

Time interval	Area of R-Isomer	% Solution stability
Initial hours	17785	**NA**
After 6 hours	17725	**99.66**
After 12 hours	17689	**99.46**
After 24 hours	17652	**99.25**
After 48 hours	17542	**98.63**

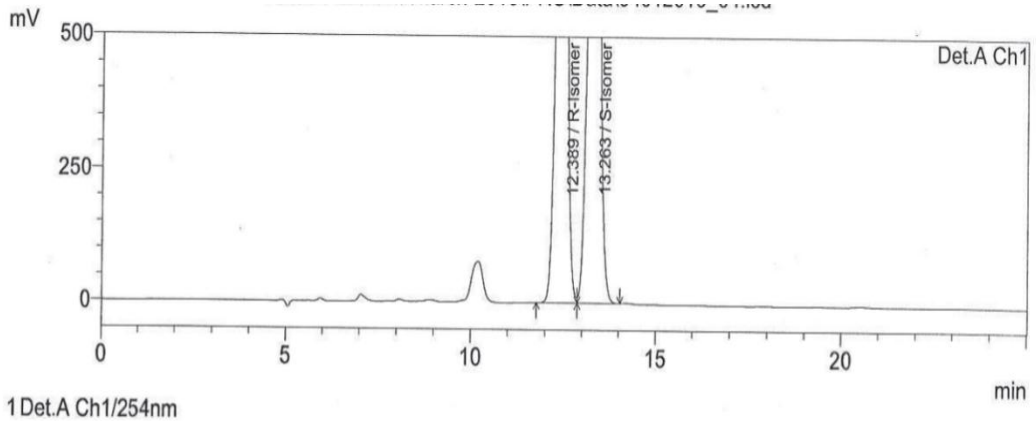


PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Area %	Name	RRT	Resolution
1	8.09	58896188	100.00	R+S Isomer	0.00	0.0
Total		58896188	100.00			

Figure 1: Chromatogram with CHIRALPAK-ADH Column (Mobile phase: MeOH: MTBE: DEA (80:20:0.1)).

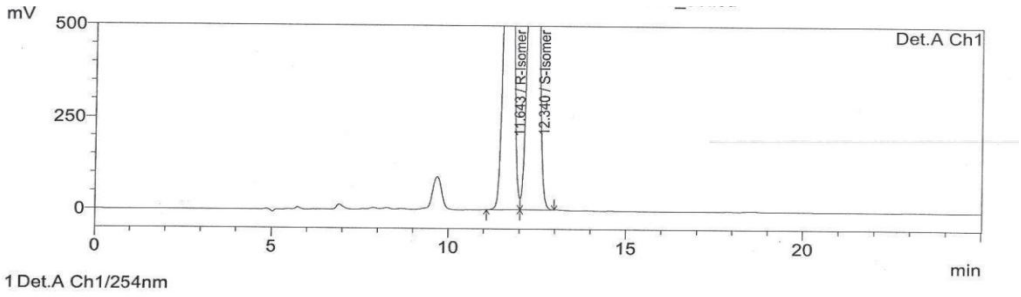


PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Area %	Name	RRT	Resolution
1	12.39	25890485	46.70	R-Isomer	1.00	0.0
2	13.26	29548976	53.30	S-Isomer	1.07	1.7
Total		55439461	100.00			

Figure 2: Chromatogram with CHIRALPAK-IE Column (Mobile phase: MeOH: MTBE: DEA (80:15:0.1)).

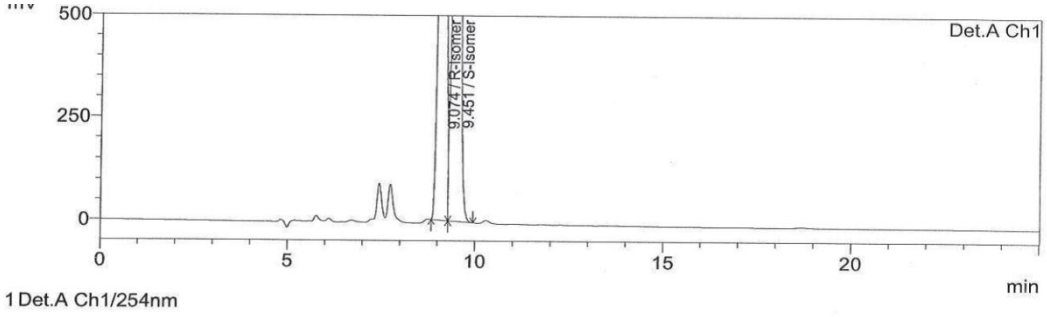


PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Area %	Name	RRT	Resolution
1	11.64	25564743	46.73	R-Isomer	1.00	0.0
2	12.34	29137936	53.27	S-Isomer	1.06	1.6
Total		54702680	100.00			

Figure 3: Chromatogram with CHIRALPAK-IE Column (Mobile phase: MeOH: MTBE: DEA (80:20:0.1)).

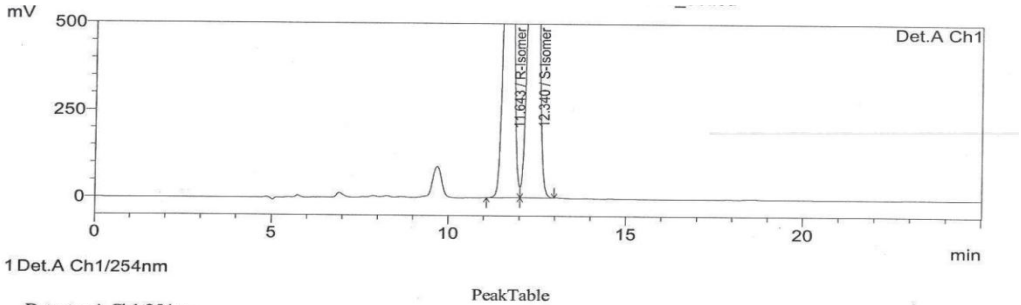


PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Area %	Name	RRT	Resolution
1	9.07	26393981	51.74	R-Isomer	1.00	0.0
2	9.45	24615860	48.26	S-Isomer	1.04	1.2
Total		51009841	100.00			

Figure 4: Chromatogram with CHIRALPAK-IE Column (Mobile phase: MeOH: MTBE: DEA (80:20:0.1)).



PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Area %	Name	RRT	Resolution
1	11.64	25564743	46.73	R-Isomer	1.00	0.0
2	12.34	29137936	53.27	S-Isomer	1.06	1.6
Total		54702680	100.00			

Figure 5: Chromatogram with CHIRALPAK-IC Column with high column temperature (30°C).

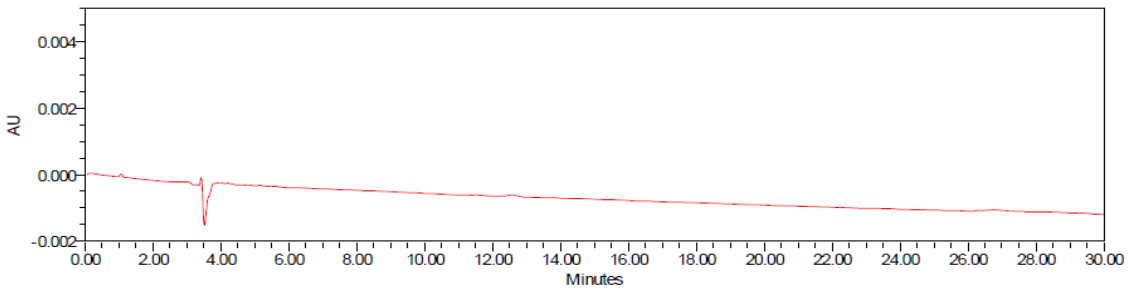


Figure 6: Chromatogram of Blank.

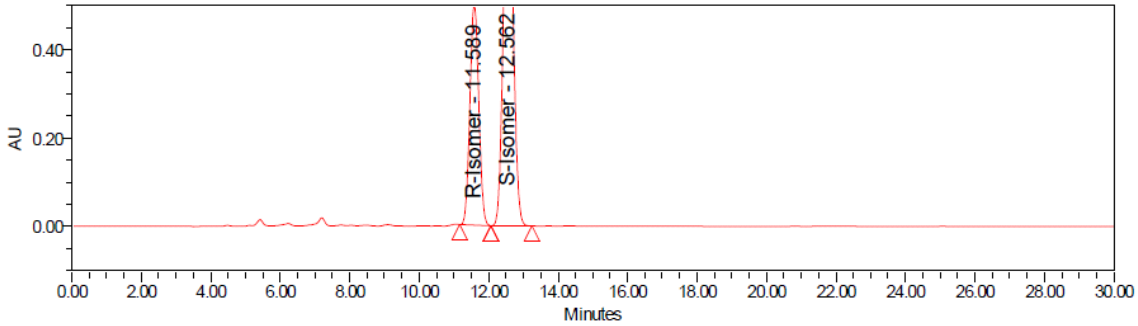


Figure 7: Chromatogram of Racemic mixture.

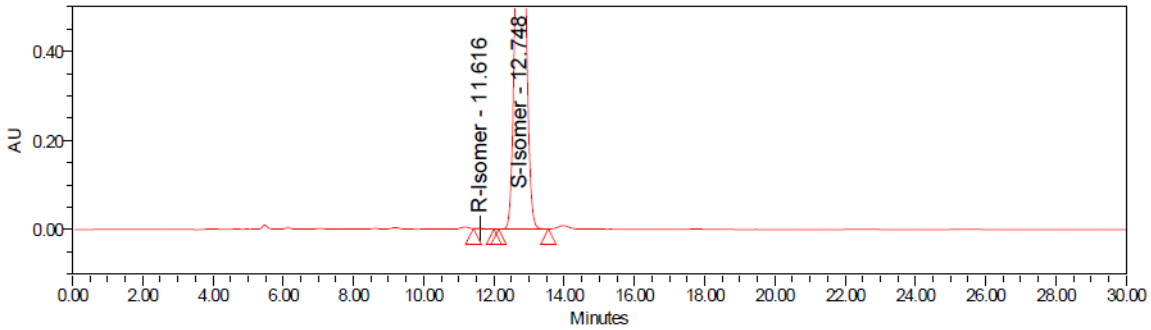


Figure 8: Chromatogram of Afatinib sample.

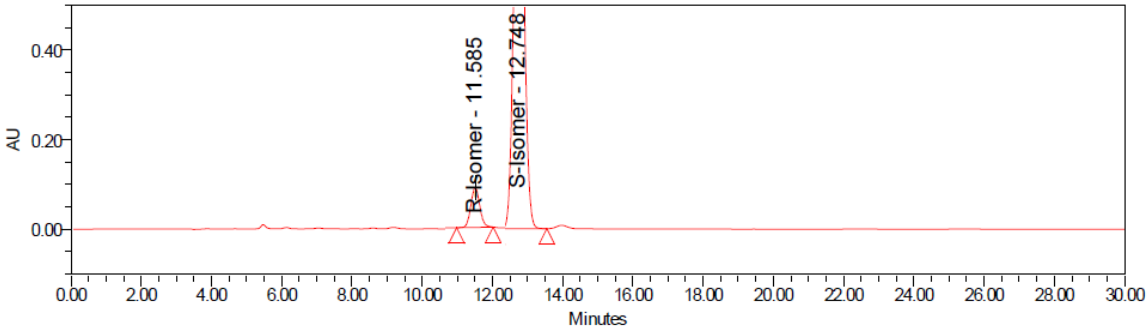


Figure 9: Chromatogram of R-Isomer spiked Afatinib sample.

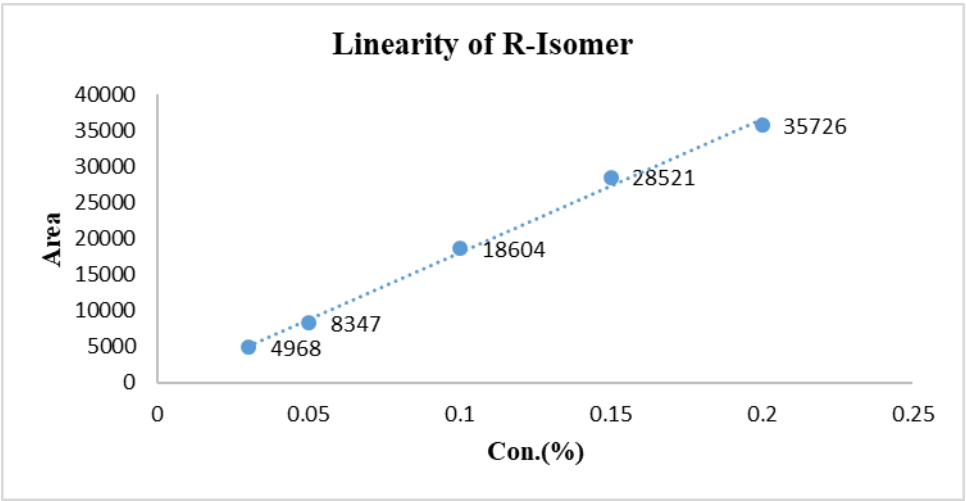


Figure 10: Calibration curve for R-isomer.

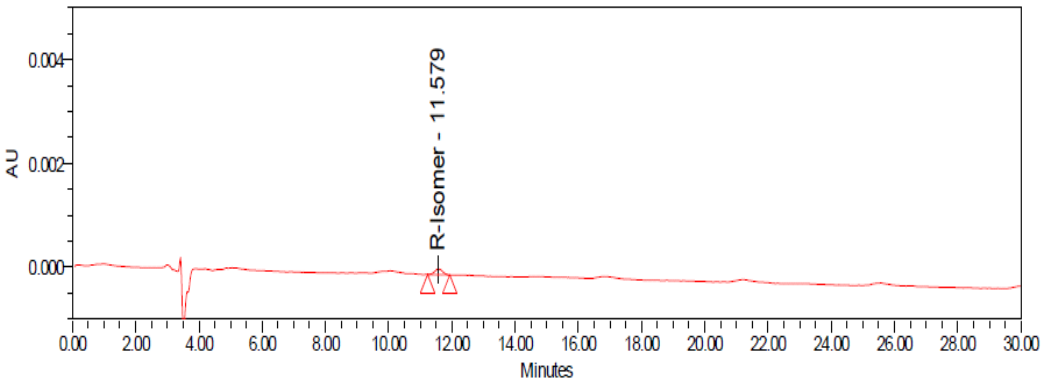


Figure 11: Representative chromatogram for LOD of R-isomer.

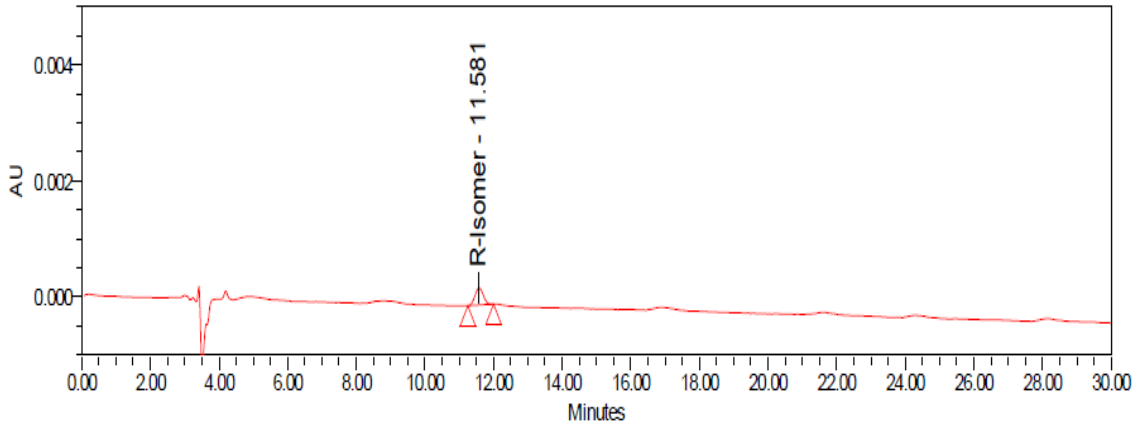


Figure 12: Representative chromatogram for LOQ of R-isomer.

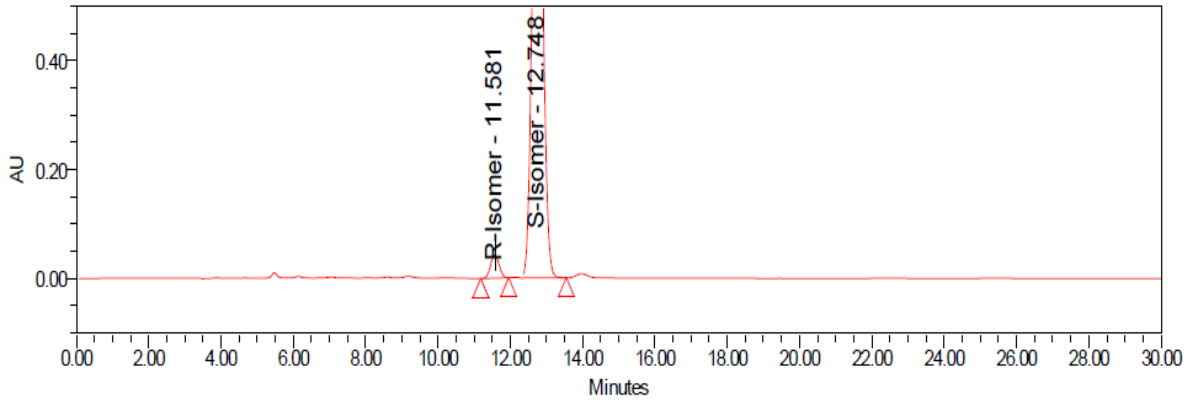


Figure 13a. Representative chromatogram of recovery for Afatinib (Recovery at 0.05%).

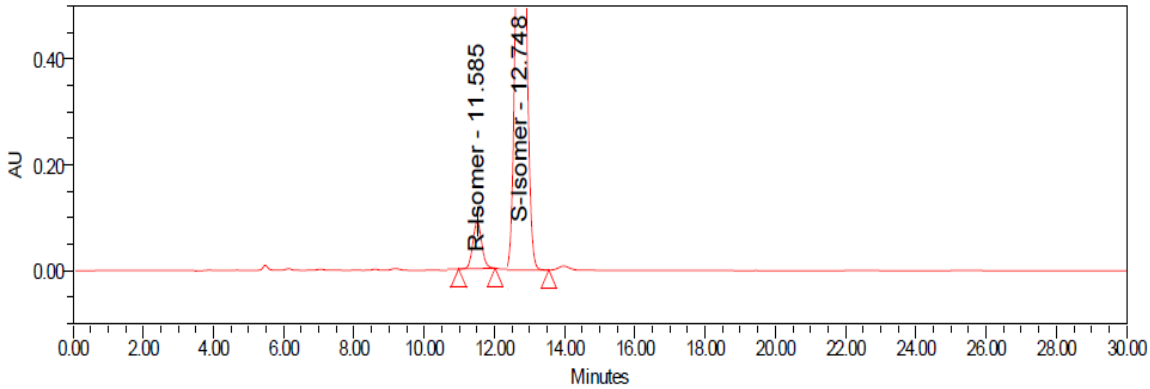


Figure 13b. Representative chromatogram of recovery for Afatinib (Recovery at 0.1%).

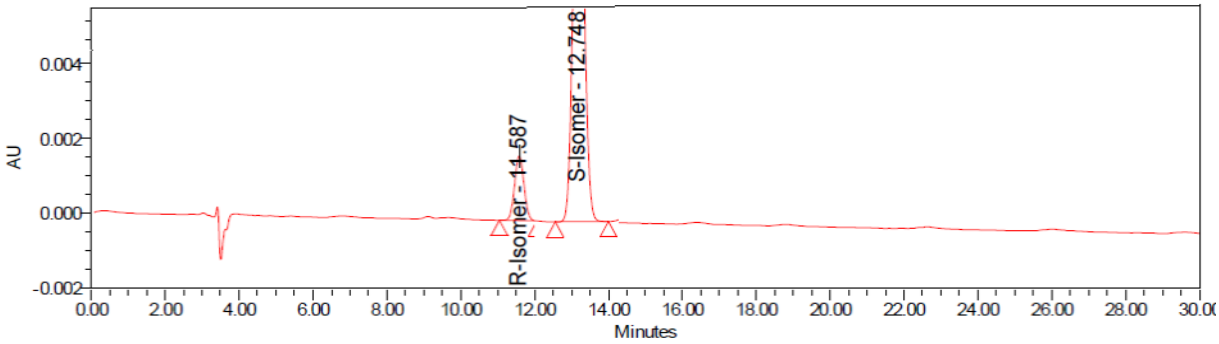


Figure 13c. Representative chromatogram of recovery for Afatinib (Recovery at 0.15%).

3.2.6. Robustness

To determine the robustness of the method, experimental conditions were purposely altered. The flow rate of the mobile phase is 0.7 mL/min. We studied the effect of flow rate on the resolution by changing from 0.6 to 0.8 mL/min while the other mobile phase components were held constant. The effect of column temperature on resolution was also studied at 15°C and 25°C, instead of 20°C while the other mobile phase components were held constant. As no significant changes in assay value were observed by changing these chromatographic conditions (flow rate and column temperature), confirms that the robustness of the method. The data of robustness are provided in table 8.

3.2.7. Solution stability of samples

Solution stability was also established for the samples and the solutions were found to be stable upto 48hrs. The representative data are provided in Table 9.

4. CONCLUSIONS

Method validation of Afatinib was carried out according to ICH Q2 guideline. In this method, both R and S-isomers were well-separated on CHIRALPAK-IE column (250 X 4.6mm X 5µ) in an isocratic elution mode with the mobile phase comprising of MeOH: MTBE: DEA (80:20:0.1; v/v) at a flow rate of 0.7 mL/ min. All the method validation parameters, recovery, linearity, precision, sensitivity and solution stability were found within the acceptable ranges. From the results, the method was found to be precise, accurate and linear from the range of LOQ (0.03%) level to 150% with respect to sample concentration and the correlation co-efficient was 0.998. Limit of detection and limit of quantifications were found to be 0.00005 and 0.00015 mg/mL, respectively. With these established data, the method was found to be very sensitive. Recovery was found to be well within the range from 95.65% to 100.73% for R-isomer. Further, the solution stability was also established and the solutions were found to be stable upto 48hrs.

5. ACKNOWLEDGEMENTS

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