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Enantioselective Analysis of Crizotinib by Chiral LC Method

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ABSTRACT

A simple, rapid, specific, precise and accurate NP-HPLC method has been developed and validated for the Separation of enantiomers of crizotinib. The chromatographic separation was achieved on chiralcel OD-H (25 × 0.46 cm, 5 µ) column at a detector wavelength of 268 nm using an isocratic mobile phase comprising a mixture of n-hexane-isopropyl alcohol-methanol- diethyl amine (40:30:30:0.5 v/v/v/v), pumped at a flow rate of 1.0 ml/min. S & R enantiomers, eluted at the retention times of 4.9 and 6.1 min respectively. The method was validated with respect to parameters such as specificity, linearity, precision, robustness, limit of detection, limit of quantification, system suitability. The developed method was found to be suitable for separation and quantification of enantiomers of crizotinib.

Keywords: Chiral separation, validation, Crizotinib

INTRODUCTION

Crizotinib [3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1Hpyrazol-4-yl)-pyridine-2-ylamine]. Crizotinib is an inhibitor of receptor tyrosine kinases, including Anaplastic Lymphoma Kinase (ALK), Hepatocyte Growth Factor Receptor (HGFR, c-Met), RON. Translocations can affect the ALK gene, resulting in the expression of oncogenic fusion proteins. The formation of ALK fusion proteins results in activation and dysregulation of the gene's expression and signaling which can contribute to increased cell proliferation and survival in tumors expressing these proteins. Crizotinib is for the treatment of ALK-positive advanced Non-small Cell Lung Cancer (NSCLC). Literature survey reveals that few HPLC Methods have been reported for the estimation of titled drug in plasma [1-5]. However, to the best of our knowledge no HPLC method is reported for chiral separation of crizotinib in bulk drug. The aim of the present work is to develop and validate a new, simple, better and economical method for the chiral separation of crizotinib enantiomers. The chemical structures of the S & R enantiomers of crizotinib are given below (Figure 1).

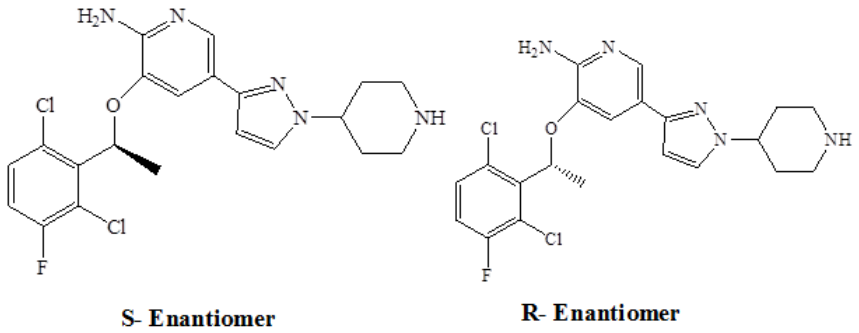


Figure 1: Chemical structures of Crizotinib

MATERIALS AND METHODS

Pure drug samples of S-Crizotinib and R-Crizotinib were purchased from Apexbio Technology LLC, Houston, USA and Bulk samples purchased from local market, (Hyderabad, India). n-Hexane, isopropyl alcohol, methanol and Diethylamine (DEA) (HPLC grade-Merck, Hyderabad) were used in the preparation of mobile phase. Mobile phase was filtered using 0.45 µm membrane filter (Millipore-Millipore Pvt. Ltd, Bangalore, India).

Instrumentation

The HPLC analysis was accomplished on Shimadzu high pressure liquid chromatograph outfitted with LC-20AD reciprocating dual HPLC pump, a manually operating Rheodyne injector with 20 µl sample loop, a 25 cm × 4.6 mm I.D analytical column (chiralcel OD-H, Tokyo, Japan) of 5 µ size and a SPD-20A model UV-Visible detector. All the parameters of HPLC were controlled by LC-Solution software. Other instruments used were Elico double beam UV-Vis spectrophotometer of model SL-210, Shimadzu electronic balance of model Aux-220.

Liquid chromatographic conditions

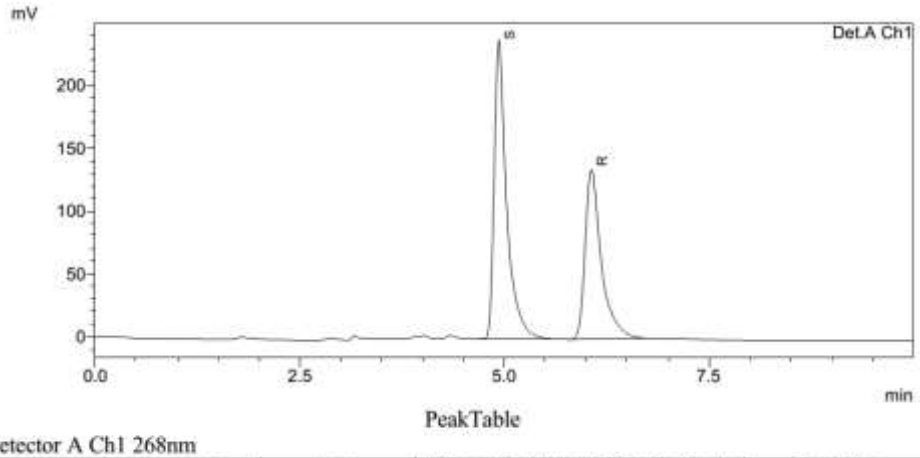
Chromatographic separation was obtained by isocratic elution mode which was performed using a mobile phase containing n-hexane-isopropyl alcohol-methanol-diethyl amine (40:30:30:0.5 v/v/v/v), at a flow rate of 1 ml/min through chiralcel OD-H (250 mm × 4.6 mm, 5 µm) column. The selective detection of the column effluent was monitored at a wavelength of 268 nm. Injection volume was 20 µl.

Preparation of stock and working mixed standard solutions

Standard stock solutions of S & R enantiomers (1 mg/ml) were prepared by dissolving accurately weighed amounts in a sufficient quantity of methanol in clean and dry 10 ml volumetric flasks. The volume was then made up to the mark with the same. Mixed working standard solutions of concentrations of, 10 µg/ml, 50, 100, 150, 200 µg/ml was prepared by appropriate dilutions of the above standard stock solutions with the diluent (mobile phase). The solutions thus prepared were filtered through 0.45 µ membrane filter and then sonicated for 5 min. Bulk sample (S and R enantiomers) solutions were prepared in the similar manner.

Method development and optimization

Optimization of the Chromatographic conditions was performed by running several trials to obtain retention times, peak symmetry, plate count, resolution within limits and possibly the best. For the selection of mobile phase, trials were done using various normal phase columns and mobile phases like, isopropyl alcohol, methanol (90:10, 80:20, 70:30, 60:40, 50:50) and n-Hexane, IPA, methanol in different ratios, tailing was observed with these conditions, a small amount of Diethylamine (DEA) increased the peak symmetry. Thus, a method using a mobile phase composition of n-hexane-isopropyl alcohol-methanol- diethyl amine (40:30:30:0.5 v/v/v/v), at a flow rate of 1 ml/min on chiralcel OD-H, 250 mm × 4.6 mm, 5 µm column at 268 nm, was found to be most suitable and satisfactory. Optimized chromatogram of S and R enantiomers was shown in Figure 2.



PeakTable

Detector A Ch1 268nm

Peak#	Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
1	4.929	2522398	4903.665	1.824	0.000
2	6.062	1854419	4417.092	1.690	3.506
Total		4376817			

Figure 2: Optimized chromatogram of S and R enantiomers

Experimental work and results

System suitability

System suitability was performed by injecting mixed standard solution of crizotinib six times in to stabilized HPLC system. The system suitability was established by evaluating parameters include retention time, repeatability, resolution (R), tailing factor (T) and theoretical plates (N). It was performed by using the concentration of 50 µg/ml. The parameters were recorded and tabulated (Table 1) and were found to be in compliance with the acceptance specifications.

Table 1: System suitability data

Name	Retention time	N	Tailing factor	Resolution	Mean peak area	%RSD
S-enantiomer	4.92	4903.66	1.82	0.00	1061189.66	**0.91**
R-enantiomer	6.06	4417.09	1.69	3.50	811969.83	**0.87**

%RSD percentage relative standard deviation

Method validation

The developed analytical method was validated as per International Conference on Harmonization (ICH) guidelines with respect to parameters

such as linearity, precision, robustness, and limit of detection, limit of quantification, and solution stability [6,7].

Linearity and range

Linearity was performed at different concentration levels i.e., working standard solutions of concentrations, 10, 50, 100, 150 and 200 µg/ml was prepared by appropriate dilutions of the standard stock solution with the diluent. The solutions thus prepared were filtered through 0.45 µ membrane filter and then sonicated for 5 min. 20 µl of each concentration was injected into the HPLC system and the corresponding chromatograms were recorded. From these chromatograms, the peak areas were calculated and linearity plots of concentration over the peak areas were constructed. The regressions of the plots were computed. Linearity results were presented in Table 2 and the calibration curves were provided in Figures 3-5 is the overlain linearity chromatogram. The linearity range was found to be 10-200 µg/ml for both (Table 3).

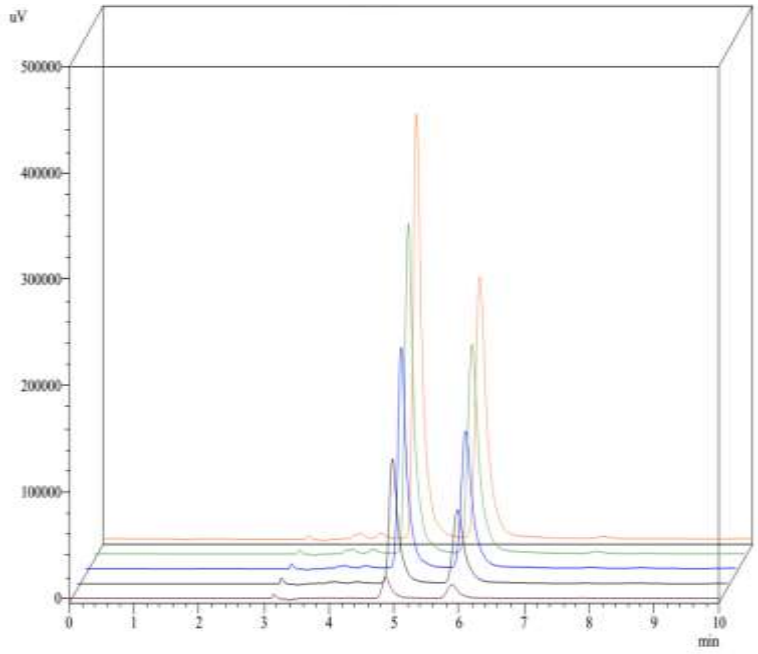


Figure 3: Overlain linearity chromatogram

Table 2: Linearity data

S. No.	Concentration (µg/ml)	S-enantiomer	R-enantiomer
		Peak area	
1	10	203012	162440
2	50	1154142	857487
3	100	2016703	1569383
4	150	3018101	2390425
5	200	3880651	2984053
Statistical analysis	Slope	92192	71762
	Correlation Coefficient (r^2)	0.999	0.998
	Y-Intercept	71124	56009

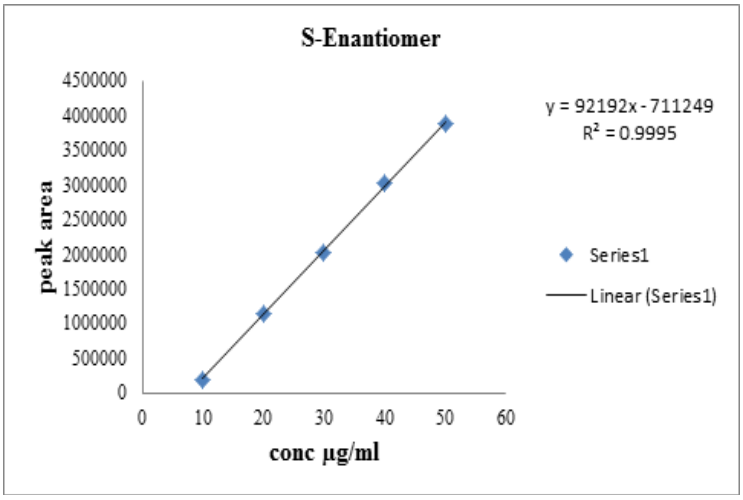


Figure 4: Calibration curve of S-Crizotinib

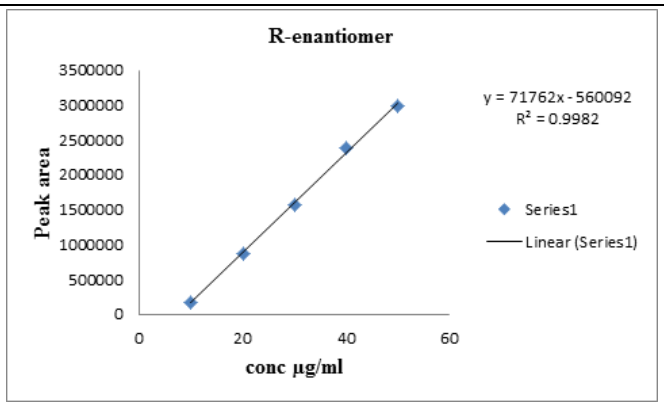


Figure 5: Calibration curve of R-Crizotinib

Table 3: Results of range

S. No.	Peak area			
	S-Enantiomer		R-Enantiomer	
	10 µg/ml	200 µg/ml	10 µg/ml	200 µg/ml
1	216831	4021593	163351	3143761
2	216036	4076173	162648	3120954
3	216025	4066325	160126	3116518
4	215048	4081709	161829	3148955
5	214711	4074134	161005	3102716
6	217086	4028599	162961	3065064
Average	215956.16	4058088.83	161986.66	3132994.66
SD	941.08	26122.12	1240.86	23376.67
%RSD	0.44	0.64	0.77	0.75

Precision

The precision was studied at the levels of repeatability (intra-day) and intermediate precision (inter-day, analyst-analyst). The intra-day precision was determined by repeating the assay on six replicate determinations (bulk drug-105 µg/ml) of S & R enantiomers, on the same day whereas the intermediate precision (100 µg/ml) was determined by repeating the same on different days and by different analysts. The % RSDs of S and R enantiomers at intra-day precision were found to be 0.41 and 0.61 respectively (Figures 6 and 7), at inter-day precision were found to be % pooled RSD were found to be 0.96 and 0.61 and at the level of analyst-analyst variation, they were found to be 0.76 and 0.57 respectively. As the %RSDs were found to be within the acceptance limit (RSD <1%) at all the levels, the proposed method was said to be precise. The results were shown in Tables 4 and 5.

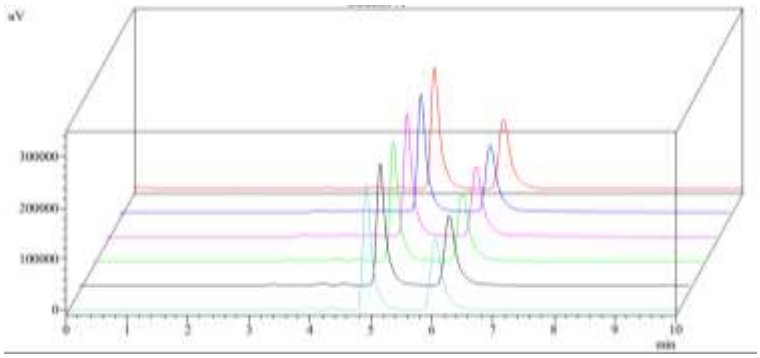


Figure 6: Overlain chromatogram of enantiomers at inter-day precision

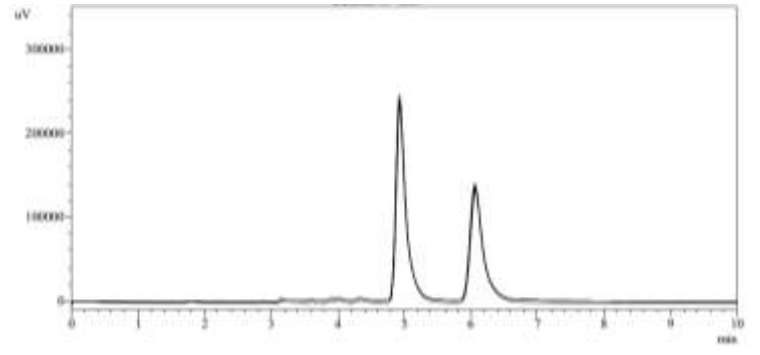


Figure 7: Overlain chromatogram of enantiomers at inter-day precision

Table 4: Intraday precision data

S. No.	S-enantiomer		R-enantiomer	
	Peak area	% Assay	Peak area	% Assay
1	2079932	103.13	1621185	103.30
2	2091277	103.60	1623538	103.45
3	2083591	103.31	1643109	104.69
4	2071653	102.72	1615409	102.93
5	2067481	102.51	1627860	103.72
6	2085503	103.41	1617512	103.06
Average		103.11		103.525
SD		0.420		0.635
%RSD		0.41		0.61

Table 5: Interday precision data of S-Crizotinib

S. No.	S – enantiomer			
	Day-1		Day-2	
	Peak area	% Assay	Peak area	% Assay
1	1940977	96.24	1962059	97.29
2	1950034	96.69	1973092	97.83
3	1919659	95.18	1956949	97.03
4	1921564	95.28	1951447	96.76
5	1927692	95.58	1964675	97.42
6	1923090	95.35	1957937	97.08
Average		95.72		97.23
SD		0.608		0.369
%RSD		0.64		0.38
Pooled %RSD=0.96%				

Table 6: Interday precision data of R-Crizotinib

S. No.	R-enantiomer			
	Day-1		Day-2	
	Peak area	% Assay	Peak area	% Assay
1	1527666	97.34	1533092	97.68
2	1525735	97.21	1516540	96.63
3	1531220	97.56	1536304	97.89
4	1526409	97.26	1513105	96.41
5	1517389	96.68	1519503	96.82
6	1513746	96.45	1541646	98.23
Average		97.08		97.27
SD		0.425		0.751
%RSD		0.44		0.77
Pooled %RSD=0.61				

Table 7: Intermediate precision data of S-Crizotinib (Analyst-analyst)

S. No.	S – enantiomer			
	Analyst-1		Analyst-2	
	Peak area	%Assay	Peak area	%Assay
1	1967522	97.56	1955439	96.96
2	1947903	96.58	1946610	96.52
3	1959902	97.18	1965310	97.45
4	1934676	95.93	1946014	96.49
5	1969882	97.67	1963404	97.35
6	1968888	97.62	1922398	95.32
Average		97.09		96.68
SD		0.70		0.77
%RSD		0.72		0.81
Pooled %RSD=0.76				

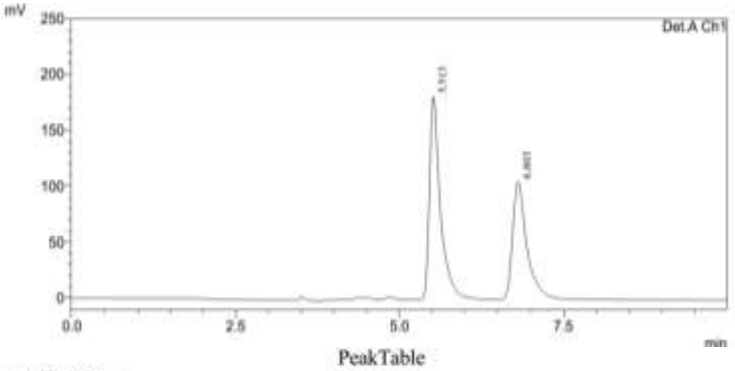
Table 8: Intermediate precision data of R-Crizotinib (Analyst-analyst)

S. No.	R-enantiomer			
	Analyst-1		Analyst-2	
	Peak area	% Assay	Peak area	% Assay
1	1527316	97.32	1525569	97.20
2	1518631	97.76	1523552	97.08
3	1519412	96.81	1528191	97.37
4	1526052	97.23	1500846	95.63
5	1516295	96.61	1528605	97.40
6	1528765	97.14	1514419	96.49
Average		97.14		96.86
SD		0.403		0.68
%RSD		0.42%		0.71
Pooled %RSD=0.57				

Robustness

Robustness of the method was demonstrated by making deliberate changes in the optimized conditions of the developed method. It was determined at 100 µg/ml. Six replicate injections were given and the effects of the variations were observed in the respectively recorded chromatograms and the %RSD of the peak areas and system suitability parameters were calculated, at each of the following conditions: Mobile phase ratio (± 2 ml); Flow rate (± 0.1 ml/min); Wavelength (± 3 nm); Concentration of DEA (-0.1 ml).

The %RSDs for S and R Crizotinib at 0.9 ml/min was found to be 0.80 and 0.96 respectively and at 1.1 ml/min they were found to be 0.77 and 0.71 respectively. At a wavelength of 265 nm, the %RSDs for the drugs were found to be 0.89 and 0.65 and at 271 nm they were found to be 0.78 and 0.92 respectively. At a mobile phase composition of n-Hexane:IPA:MeOH in a ratio of 38:30:32, the %RSDs for S and R crizotinib were found to be 0.86 and 0.60 respectively and at a ratio of 42:30:28, they were found to be 0.68 and 0.82 respectively. As the %RSDs (< 1%) of peak area and system suitability parameters were found to be within the acceptance limits. No significant deviations were observed which indicate that the method was robust. The results were shown in Figures 8, 9 and Tables 6-9.

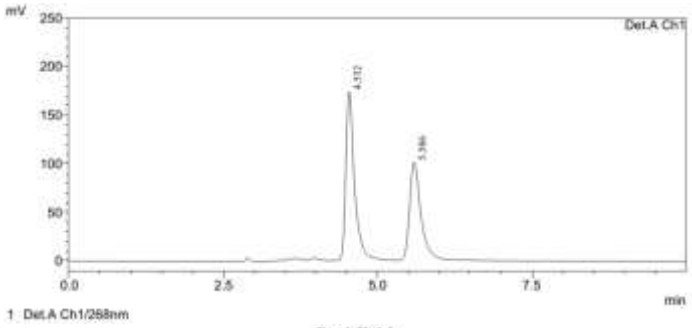


PeakTable

Detector A Ch1 268nm

Peak#	Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
1	5.513	2100795	5108.981	1.833	0.000
2	6.803	1573695	4686.756	1.684	3.656
Total		3674490			

Figure 8: Chromatogram of S & R enantiomers at 0.9 ml/min (Robustness study)



PeakTable

Detector A Ch1 268nm

Peak#	Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
1	4.532	1686351	4879.276	1.681	0.000
2	5.586	1274444	4394.625	1.552	3.533
Total		2960795			

Figure 9: Chromatogram of S & R enantiomers at 1.1 ml/min (Robustness study)

Table 9: Robustness study results

Parameter		S-enantiomer		R-enantiomer	
		Mean peak area	**% RSD**	**Mean peak area**	**% RSD**
Mobile phase ratio n-Hexane:IPA:MeOH (± 2 ml)	38:30:32	1921783.83	0.86	1443665.83	0.60
	40:30:30	1848021.16	0.71	1350499.83	0.88
	42:30:28	1952422.33	0.68	1451472.83	0.82
Flow rate (ml/min)	0.9	2104251.33	0.80	1568715.83	0.96
	1.0	1848021.16	0.71	1350499.83	0.88
	1.1	1745271.0	0.77	1245056.33	0.71
Wavelength (± 3 nm)	265	1851892.0	0.89	1371324.0	0.65
	268	1848021.16	0.71	1350499.83	0.88
	271	1856506.83	0.78	1417686.5	0.92
DEA % (ml)	0.4	1939126.16	0.80	1460226.33	0.47
	0.5	1848021.16	0.71	1350499.83	0.88

LOD and LOQ

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessary quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of an analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Demonstrated the limit of detection and quantification based on the standard deviation of intercept (σ) and slope (S) of linearity data, obtained by preparing the diluted standard solutions in the range of 1-5 µg/ml. The lowest possible concentrations of S & R enantiomer that can be detected by the proposed method were found to be 0.33 µg/ml and 0.30 µg/ml respectively. The lowest possible concentrations of S & R enantiomers that can be quantified by the proposed method were found to be 1.0 µg/ml and 0.92 µg/ml respectively. The results are given in table. The following formulas were used to calculate LOD and LOQ (Table 10).

$$\text{LOD} = \frac{3.3\,\sigma}{S} \quad \text{LOQ} = \frac{10\,\sigma}{S}$$

Table 10: LOD and LOQ data

S- Crizotinib			R- Crizotinib		
Concentration (µg/ml)	**Peak area**	**Statistical analysis**	**Concentration (µg/ml)**	**Peak area**	**Statistical analysis**
1	44369	σ=3238.758	1	37436	σ=2446.103
2	77760	S=32095.9	2	68885	S=26434
3	113580		3	92666	
4	144653	LOD=0.33 (µg/ml)	4	117017	LOD=0.30 (µg/ml)
5	171402	LOQ=1.0 (µg/ml)	5	145540	LOQ=0.92 (µg/ml)

σ=Standard deviation of Y-Intercepts, S=Slope of the calibration curve

Stability

Solution stability was estimated for S and R-enantiomers along with mobile phase stability. The solution was injected after their preparation and the peak area values were recorded (and percentage of drugs were calculated) and kept the solution at room temperature. For every 12 h, this solution was injected along with the freshly prepared solutions, and the peak areas were recorded. The same procedure was repeated, until there was a significant change (due to degradation) in the drug concentration. The solution stability studies were performed and the drug solutions were found to be stable for 2 days (48 h) from the time of their preparation at room temperature and mobile phase was stable for up to 3 days at 20°C.

CONCLUSION

A simple and rapid Normal-phase HPLC method was developed and validated according to ICH guidelines for the chiral separation and determination of S and R enantiomers of Crizotinib in bulk. The developed method was proved to be simple, rapid, robust, reproducible and economical. Because of its simplicity and low cost, the method can be used for routine practices in the laboratories.

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