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Validated enantiospecific LC method for determination of (*R*)-enantiomer impurity in (*S*)-efavirenz

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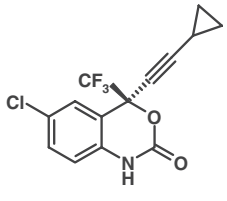
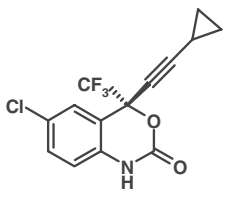
A high-performance liquid chromatographic method was developed for separation of the enantiomers of efavirenz. The developed method was applied for the determination of (*R*)-enantiomer in (*S*)-efavirenz and satisfactory results were achieved. The base line separation with a resolution of more than 4.0 was achieved on Chiralcel OD (250 mm × 4.6 mm, 10 μm) column containing tris-(3,5-dimethylphenylcarbomate) as stationary phase. The mobile phase consists of *n*-hexane: isopropyl alcohol (80 : 20 v/v) with 0.1% (v/v) of formic acid as additive. The flow rate was kept at 1.0 ml/min and the UV detection was monitored at 254 nm. The (*R*)-enantiomer was found linear over the range of 0.1 μg/ml – 6 μg/ml. The limit of detection (LOD) was 0.03 μg/ml and the limit of quantification (LOQ) was 0.1 μg/ml (n = 3). The precision of (*R*)-enantiomer at LOQ level was evaluated through six replicate injections and the RSD of the peak response was achieved as 1.34%. The results demonstrated that the developed LC method was simple, precise, robust and applicable for the purity determination of efavirenz.

1. Introduction

Efavirenz is a specific, non-nucleoside reverse transcriptase inhibitor (NNRTI) for treatment of human immunodeficiency virus type 1 (HIV-1) (Adkins and Noble 1998; Young et al. 1995). The drug molecule contains one chiral center and the absolute configuration of the molecule is *S*. Few analytical methods reported in literature for determination efavirenz and its related substances in drug substance and in a capsule formulation (Montgomery et al. 2001), investigation of critical factors for the resolution of key impurity in dosage forms (Weissburg et al. 2002), impurity profiling on graphite carbon stationary phase (Xu et al. 2003). The methods reported are directly related to the determination of organic impurities. One method was reported (G. Lavison et al. 2003) for the determination of enantiomers content on ristocetin bonded stationary phase. This method is based on Supercritical Fluid Chromatography (SFC) and use of a highly expensive column, where the column efficiency was reported as 2900 theoretical plates and the resolution between the two enantiomers was of 2.6. We are describing here a conventional liquid chromatographic technique with inexpensive columns. The study was focused on determination of enantiomeric purity with a target of 0.1% quantification of (*R*)-enantiomer in the drug substance (API), (*S*)-efavirenz.



Efavirenz (S-Form)



R-Enantiomer

2. Investigations, results and discussion

2.1. Column selectivity and method optimization

Two different polysaccharide-based stationary phase columns, Chiralcel OD (Cellulose tris (3,5-dimethylphenylcarbamate)) and Chiralcel OJ (cellulose tris(4-methylbenzoate)) were evaluated for method development activity, using isopropyl alcohol and ethanol as organic modifiers in *n*-hexane. The chiral recognition mechanism on these chiral stationary phases (CSPs) is generally due to the formation of solute-CSP complexes through inclusion of enantiomers into the chiral cavities in the higher order structures of the CSPs (Okamato and Yashima 1998; Yashima et al. 1998; Okamato and Kaida 1994). The carbomate groups can interact with solutes through hydrogen bonding using C=O and N-H groups, and through dipole-dipole interaction using C=O moiety. Efavirenz contains a functional N−H group and can form hydrogen bonds with C=O group in the CSPs. A Chiralcel OJ column did not show selectivity for efavirenz enantiomers with isopropyl alcohol and ethanol as organic modifiers in *n*-hexane. However, little selectivity (resolution of 1.26) was shown while using a Chiralcel OD column with 10% isopropyl alcohol in *n*-hexane. A higher resolution was obtained with 20% isopropyl alcohol. Formic acid was added (0.1%) to the mobile phase for better peak shapes. Finally

Table 1: System suitability parameters

Compound (n = 3)	RT	R	N	T	α
Efavirenz	10.944	–	7495	1.06	–
(*R*)-Enantiomer	13.536	4.59	8063	1.06	2.52

RT: Retention time in minutes; R: Resolution; N: No. of theoretical plates; T: Tailing factor; α: Selectivity

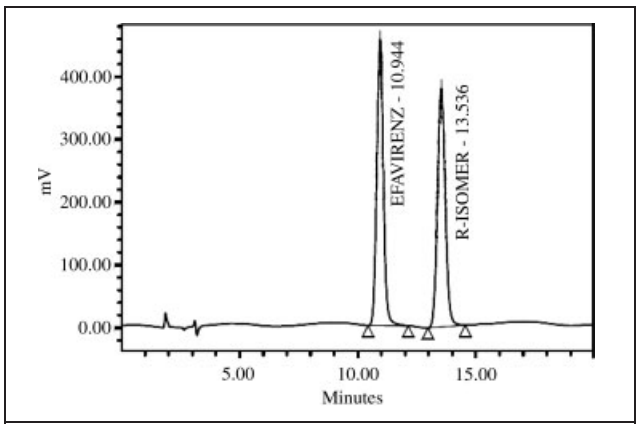


Fig. 1: Typical chromatogram of enantiomeric separation of efavirenz

the method was optimized using a mobile phase containing *n*-hexane : isopropyl alcohol : formic acid (80 : 20 : 0.1 v/v/v). Column temperature was maintained at 25 °C.

2.2. Method validation

2.2.1. System suitability

Under the conditions described in section 3.3, good chromatography parameters were obtained, with a resolution of more than 4.0 between the peaks of (*R*)- and (*S*)-efavirenz. The number of theoretical plates of the column was more than 7000. The results are presented in Table 1 (system suitability parameters). The chromatogram pertaining resolution of (*R*)- and (*S*)-isomers is depicted in Fig. 1. The figure indicates that the (*R*)- and (*S*)-isomers were separated completely up to base line under the conditions employed.

2.2.2. Precision

The precision of the developed method was evaluated by analyzing six test solutions of enantiomers prepared at LOQ level. Peak response corresponding to each test solution was measured and relative standard deviation was calculated. The relative standard deviation was 1.34%, which confirms that the method was sufficiently precise.
Intermediate precision was evaluated by injecting different preparations of system suitability solutions and calculating the relative standard deviations of retention times and peak responses. The relative standard deviations of retention times and peak responses are calculated as $<1.0\%$ in both cases for both efavirenz and its (*R*)-enantiomer.

2.2.3. Linearity and range of (R)-enantiomer impurity

Detector's linear response was evaluated by preparing six calibration solutions of (*R*)-enantiomer over the concentration range of LOQ (0.1 μg/ml) to 120% (6.0 μg/ml).
Calibration curve was constructed by plotting peak area versus concentrations of (*R*)-enantiomer. The regression equation was calculated as $y = 8746.76 \times + 46.9$ and the corresponding correlation coefficient of linear regression equation was 0.9999. The measured response of known standards in the linearity range was entered in the above regression equation and calculated the concentrations. The residuals (difference between observed concentration and predicted concentrations) were found distributed and centered around zero.
The regression equation and correlation coefficients indicates that the analytical method is linear over the range 0.1−6 μg/ml of investigation. The details of validation results are presented in Table 2.

Table 2: Validation results of analytical method

Validation parameter	Result
Linearity of enantiomer	
Calibration range	0.10 μg–6.0 μg
Calibration points	6
Correlation coefficient	0.9999
Slope (% RSD)	0.03
Intercept (% RSD)	4.72
LOD & LOQ data	
Limit of detection	0.03 μg/ml
Limit of Quantification	0.10 μg/ml
Precession at LOQ (% RSD)	1.34%
Intermediate precision data (% RSD)	
Retention time (enantiomer)	0.16
Retention time (efavirenz)	0.68
Peak area (enantiomer)	0.18
Peak area (efavirenz)	0.09

2.2.4. Accuracy

The standard addition and recovery experiments were carried out for (*R*)-enantiomer in efavirenz bulk sample in triplicate at concentration levels of 0.1%, 0.25% and 0.5% of analyte concentration (analyte concentration was set at 0.5 mg/ml). The recoveries were found to be in the range of 98.28% to 99.9% with the RSD values in the range of 0.4% to 1.43%.

2.2.5. Robustness

Robustness of the method was studied for the resolution between the enantiomers by making small but deliberate changes in method parameters. A variation of about 5% in isopropyl alcohol content in the mobile phase composition had no influence on resolution except for changes in retention times. The effect of temperature was studied with the column temperature variation of ±2 °C and it was observed that the resolution was not affected due to variations in column temperature. A third type of robustness study was carried out using changes in mobile phase flow at 0.9 ml/min and 1.1 ml/min. The resolution between the enantiomers was not influenced by in the changes in the robustness study. The resolution between the enantiomers was higher than 4.0 in all cases. The variations in retention times observed without any impact on resolution between the enantiomers. Details of the robustness study are presented in Table 3.

2.2.6. Limit of detection and limit of quantitation

The limits of detection (LOD) and quantification (LOQ) were calculated using the signal to noise (S/N) ratio method. LOD was taken as a concentration of the analyte where S/N

Table 3: Robustness data

Parameter altered	Resolution
Flow rate	
0.9 ml/min	4.27
1.0 ml/min	4.56
1.1 ml/min	4.16
Column temperature	
23 °C	4.32
25 °C	4.56
27 °C	4.47
Organic modifier (in percent)	
15	4.47
20	4.56
25	4.61

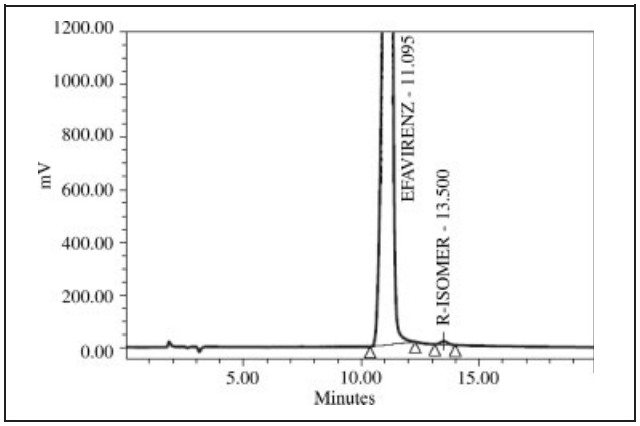


Fig. 2: Typical chromatogram of efavirenz bulk sample spiked with enantiomer

was about 3 and was estimated as 0.02 μg/ml and 0.03 μg/ml for efavirenz and its (*R*)-enantiomer, respectively.
LOQ was taken as a concentration of the analyte where S/N was about 10 and was estimated as 0.06 μg/ml and 0.10 μg/ml for efavirenz and its (*R*)-enantiomer, respectively.

2.3. Application of the method for analysis of bulk samples

The bulk sample of efavirenz provided by process research laboratory of Matrix laboratories had an (*R*)-enantiomer content below detection limit. Standard addition and recovery experiments were conducted to determine the accuracy of the analytical method for quantification of the (*R*)-enantiomer content in bulk samples.
The study was carried out in triplicate at 0.1, 0.25 and 0.5% of the efavirenz target analyte concentration. The recovery was calculated from the slope and intercept of the calibration curve obtained in a linearity study and was in the range of $100 \pm 2\%$. A typical chromatogram of bulk sample spiked with 0.1% is presented in Fig. 2.

3. Experimental

3.1. Materials and reagents

All reagents used during the analysis were of analytical regent grade unless stated otherwise. HPLC grade *n*-hexane and isopropyl alcohol were purchased from JT Beker, USA. Formic acid was procured from Ranbaxy fine chemicals, Mumbai, India. Qualified standards of efavirenz (99.99% purity) and its (*R*)-enantiomer (99.97% purity) were obtained from antiviral research laboratory of Matrix Laboratories Limited, India.

3.2. Apparatus

All analysis and development activities were performed with a Waters HPLC system consisting of a 2695 separation module, a 2487 dual wavelength UV detector, auto sampler, column heater, degasser and sample cooler. Waters Empower software was used for data acquiring and calculation of system suitability parameters.

3.3. Chromatographic conditions

Chromatographic separation was achieved on Chiralcel OD (250 mm × 4.6 mm; particle size 10 μm, procured from M/s. Diacel Industries, USA) column attached with Chiralcel OD guard column. The mobile phase consisted of *n*-hexane : isopropyl alcohol : formic acid (80 : 20 : 0.1 v/v/v). The flow rate was maintained at 1.0 ml/min and the UV detection was kept at 254 nm for monitoring of elutes. Column temperature was maintained at 25 °C with injection volume of 20 μl. Total run time of the analysis was 30 min.

3.4. Preparation of stock and standard solutions

Stock solutions of efavirenz and its enantiomer were prepared by dissolving appropriate quantities in mobile phase to attain concentrations of about 10.0 mg/ml of both solutions. These stock solutions were refrigerated during validation study.
Test solution was prepared by diluting the stock solution to attain concentration of 5 mg/ml. Standard solutions were prepared by diluting appropriate volumes of stock solutions in mobile phase to attain required concentrations for linearity/range (0.1 to 6.0 μg/ml).

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