Journal of Food and Drug Analysis

Volume 21 | Issue 1 Article 5

2013

Chiral separation of non-nucleoside reverse transcription inhibitor efavirenz by HPLC on cellulose-based chiral stationary phase

Journal of Food and Drug Analysis, Vol. 21, No. 1, 2013, Pages 93-100 doi:10.6227/jfda.2013210112

Chiral Separation of Non-Nucleoside Reverse Transcription Inhibitor Efavirenz by HPLC on Cellulose-Based Chiral Stationary Phase

S. S. PUJERI[1], A. M. A. KHADER[1] AND J. SEETHARAMAPPA[2]*

[1.] *Department of Chemistry, Mangalore University, Mangalagangotri, India*
[2.] *Department of Chemistry, Karnatak University, Dharwad, India*

(Received: April 8, 2012; Accepted: August 29, 2012)

ABSTRACT

A stereospecific HPLC method for the separation of efavirenz (EFZ) enantiomers in bulk drug and formulations was developed and validated on a normal-phase cellulose derivatized chiral column. The effect of organic modifiers, namely, 2-propanol, ethanol and trifluoroacetic acid in mobile phase was optimized to obtain the best enantiomeric separation. The retention times of (R)-EFZ and (S)-EFZ were observed to be 7.5 and 9.2 min, respectively. The calibration curve was found to be linear over the concentration range of 200 - 6210 ng/mL with a determination coefficient (R^2) of 0.9999 for the (R)-isomer. The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 66 ng/mL and 200 ng/mL, respectively. The proposed method was found to be accurate, precise and suitable for the separation and quantification of unwanted (R)-EFZ in active pharmaceutical ingredients (API). The analytical results were supported by statistical parameters. The proposed method could be successfully applied to the enantiomeric purity analysis of EFZ in bulk drug samples and formulations.

Key words: efavirenz, enantiomeric separation, HPLC, cellulose-based stationary phase, validation

INTRODUCTION

The United States Food and Drug Administration, European Committee for Proprietary Medicinal Products and other drug controlling agencies have issued marketing guidelines for optically active pure drugs and hence, the demand for chiral separations has increased greatly[(1,2)]. Nowadays, the separation of enantiomers has become an essential and important step, particularly in pharmaceutical and biological fields, since some enantiomers of racemic drugs have relatively different pharmacokinetic properties and diverse pharmacological or toxicological effects[(3-5)]. This is one of the most vital reasons why the regulatory authorities insist on the evaluation of the safety and effectiveness of drugs containing chiral centers. Enantiomeric separations have acquired importance in all the stages of drug development and the commercialization process. Therefore, the development of new methods for efficient chiral separations based on HPLC, capillary electrophoresis (CE) or gas chromatography (GC) assume importance.

Efavirenz (EFZ), administered as a single enantiomer, (S)-6-chloro-4-(cyclopropylethynyl)-l,4-dihydro-4-(trifluoromethyl)-2H-3,l-benzoxazin-2-one (Figure 1), is a specific, non-nucleoside reverse transcriptase inhibitor (NNRTI) of the human immunodeficiency virus type 1 (HIV-1)[(6)]. Its long half-life allows once-daily dosing, and therefore presents an advantage for treatment compliance and efficacy[(7-9)]. EFZ undergoes extensive metabolism, mainly by the cytochrome P-450 isoenzyme, CYP2B6, which is known to exhibit extensive inter-individual variability. This could lead to heterogeneity in response to treatment.

A number of high performance liquid chromatographic methods have been reported for the assay of EFZ in plasma

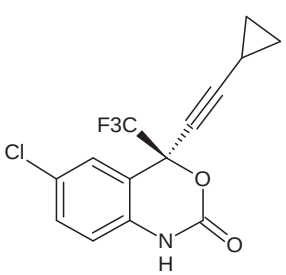

Figure 1. Structure of (S)-EFZ.

* Author for correspondence. Tel: +91-836- 2215286/27; Fax: +91-836-2747884; Email: jseetharam@yahoo.com

alone[10-15] and in combination with other antiviral agents[16-28]. Critical literature survey showed that no chiral HPLC method has been reported for the separation of the enantiomers of EFZ. In this study, a simple and efficient chiral HPLC was developed and validated for the separation of enantiomers of EFZ with commercially available chiral stationary phase (Chiralcel OD-H). The developed method is successfully applied for the evaluation of enantiomeric purity in bulk and pharmaceutical formulations. Further, the method is sensitive as evident from the low detection limit of 65 ng/mL for (R)-EFZ.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

The racemic mixture of EFZ was obtained from Arobindo Pharmaceuticals (Hyderabad, India). (R)-EFZ (chemical purity:99.85%; chiral purity:99.51%) and (S)-EFZ (chemical purity:99.89%; chiral purity:99.91%) were obtained by purifying the racemic mixture and bulk drug, respectively, on preparative HPLC. EFZ tablets (Sustiva 600 mg film-coated tablets) were obtained commercially. N-Hexane (HPLC grade) was purchased from Spectrochem Pvt. Ltd. (Mumbai, India). 2-propanol was purchased from Merck (Mumbai, India). Ethanol (absolute) was obtained from Tedia Company Inc. (Fairfield, OH, USA). Trifluoroacetic acid was purchased from Aldrich Chemicals Co., Inc. (Milwaukee, WI, USA).

II. *Instrumentation*

The equipment consisted of a Waters 2695 separation module system consisting of an online degasser, quaternary pump, auto-sampler equipped with cooling system, thermostat column compartment and 2996 module PDA (photo diode array) detector (Waters Corp., Milford, MA, USA). The output signal was monitored and integrated using Waters Empower software (Build 1154). The Chiralcel OD-H column (250 mm × 4.6 mm, 5.0 µm) (Diacel Chemical Industries, Tokyo, Japan) was used in the study. For method development, Chiralpak AD, AD-H and AD-RH, Chiralcel OD, OD-H and OD-RH, Chiralcel OJ and OB columns were used (Diacel Chemical Industries Tokyo, Japan). The mobile phase and sample/standard preparations were degassed by using a sonicator (S.V. Scientific, India).

A preparative HPLC system consisting of an Agilent 1200 series (CA, USA) binary pump, auto sampler, PDA detector and fraction collector was used for obtaining the standards of (R)-Efavirenz and (S)-Efavirenz using a semi-preparative Chiralcel-OD column (250 mm × 10 mm, 10.0 µm) (Diacel Chemical Industries, Tokyo, Japan).

III. *Chromatographic Conditions*

The mobile phase was prepared by mixing n-hexane, 2-propanol and trifluoroacetic acid in the ratio of 90 : 10 : 0.05 (v/v/v) and degassed using a vacuum degasser before use. The flow rate was set at 0.8 mL/min and the column was maintained at ambient temperature. The injection volume was 10 µL and the detector wavelength was tuned at 245 nm. An n-hexane and 2-propanol mixture in the ratio of 90 : 10 (v/v) was used as diluent for sample and standard preparation and the same was injected as blank. The mobile phase and sample/standard preparations were degassed using a sonicator.

The column was flushed with a mixture of n-hexane and 2-propanol (90 : 10, v/v) at the end of each day at a flow rate of 0.4 mL/min for 2 h in order to regenerate after using the mobile phase containing TFA.

For preparative HPLC, a mobile phase consisting of a mixture of n-hexane, 2-propanol and trifluoroacetic acid in the ratio of 90 : 10 : 0.05% (v/v/v) was pumped at a flow rate of 3.0 mL/min on a semi-preparative Chiralcel-OD (250 mm × 10 mm) column at ambient temperature. A 1-mL aliquot of a 15 mg/mL solution of the racemic mixture of EFZ was injected and the eluent was monitored at 245 nm.

IV. *Procedures*

(I) *Sample Preparation*

Bulk drug: 20 mg of the bulk drug EFZ sample was weighed into a 10-mL volumetric flask. 2 mL of 2-propanol was added to dissolve the bulk drug with sonication. The volume was made up to the mark with the diluent and the solution was filtered through a 0.2-µm syringe filter.

Tablets: Five tablets of EFZ were pulverized and weighed. The powder equivalent to 10 mg of EFZ was weighed and transferred into a 10-mL volumetric flask. 2 mL of 2-propanol was added and the mixture was sonicated for 15 min. 3 mL of diluent was added and the mixture was sonicated for a further 10 min. The volume was finally made up to the mark with the diluent and the solution was filtered through a 0.2-µm syringe filter. It was further diluted as required for analysis.

(II) *Specificity*

The specificity of the method was established through a study of the resolution factors of the enantiomer peaks from the nearest resolving peak and also among all the other peaks due to excipients, impurities and degraded products[29]. System suitability parameters were calculated.

(III) *Limits of Detection (LOD) and Quantification (LOQ)*

The limits of detection and quantitation of EFZ enantiomers were estimated by obtaining the detector signals for the peaks and by performing serial dilution of a solution of known concentration. The values of LOD and LOQ were determined at a signal to noise ratio of 3 : 1 and 10 : 1, respectively. The LOQ value was determined by injecting a series of test solutions of known concentrations within the linearity

range. Precision study was also carried out at the LOQ level by injecting six preparations.

(IV) *Precision*

The repeatability of the method was evaluated by calculating the RSD of the area of the unwanted (R)-EFZ in spiked samples for six replicate injections. Further, the RSD values were calculated by carrying out the analysis on three consecutive days, six times in each day.

(V) *Linearity and Range*

The test solutions were prepared from the stock solution of EFZ so as to contain the drug in the range of 200 - 6210 ng/mL. The solutions were injected in triplicate into the HPLC column, using a constant injection volume of 10 µL and the chromatograms were recorded. The standard drug was spiked with (R)-EFZ at different concentrations and injected in triplicate. The peak area *versus* concentration data was treated by least-squares linear regression analysis. Chromatograms of the above test solutions were recorded for three consecutive days in the same concentration range. The percentage RSD values were calculated.

(VI) *Accuracy*

The accuracy of the method was evaluated by spiking the standard drug with known amounts of the unwanted (R)-EFZ at LOQ level, 0.8, 1.0 and 1.2% of analyte concentration in triplicate. The recoveries were calculated from the slope and the intercept obtained for the calibration curve of the (R)-EFZ standard.

(VII) *Robustness*

To determine the robustness of the method, the experimental conditions were purposely altered and chromatographic resolution between (S)-EFZ and (R)-EFZ was evaluated. To study the effect of flow rate on the resolution of enantiomers, it was varied between 0.6 to 1.0 mL/min with 0.2-unit increments. Further, to examine the effect of the organic modifier (trifluoroacetic acid) on the resolution of enantiomers, its composition was fixed at 0.04 and 0.06% in the mobile phase and the chromatograms were recorded. The influence of the organic solvent, 2-propanol, in the mobile phase on resolution was studied by varying it from -1 to +1% and keeping other mobile phase components constant. The % RSD value (n=3) for the resolution between (S)-EFZ and (R)-EFZ was calculated.

(VIII) *Solution Stability*

Standard solutions of (S)-EFZ and (R)-EFZ were prepared in the mobile phase at analyte concentration levels. Each standard solution was analyzed immediately after preparation and divided into two parts. One part was stored between 2 - 8°C in a refrigerator and the other on the bench top in tightly capped volumetric flasks. The stored solutions of each isomer were analyzed up to 24 h with an interval of every 6 h.

The mobile phase stability was carried out by evaluating the content of (R)-EFZ in EFZ sample solutions spiked with (R)-EFZ at different levels, prepared freshly at 6 h intervals for 24 h. The same mobile phase was used during the study. The percentage RSD values were calculated for the mobile phase and solution stability experiments.

RESULTS AND DISCUSSION

I. *Method Development*

To achieve separation between the enantiomers of EFZ, immobilized polysaccharide chiral stationary phases (CSPs) containing cellulose and amylose derivatives were examined with suitable mobile phase compositions. The chiral discrimination of enantiomers occurs when they bind to the stationary phase forming transient diastereomeric complexes. The most important interactions between the analyte and CSP include hydrogen bonding, dipole-dipole interactions and pi-pi interactions, together with the rigid structure (cellulose-based CSP; 1, 4-connected-β-D-glucose unit) or helical structure (amylose-based CSP; 1, 4-connected-α-D-glucose unit) of the chiral polymer bound to the support. Various combinations of n-hexane-ethanol and n-hexane-2-propanol, in the absence and presence of TFA, were used as the mobile phases in the initial trials. Attempts to separate the enantiomers on amylose carbamate derivatized columns in normal phase (Chiralcel AD and AD-H) proved futile. The enantiomers could not be resolved satisfactorily even with the cellulose ester derivatized columns (Chiralcel OJ and Chiralcel OB).

The separation was attempted in reversed phase using cellulose and amylose carbamate derivatized columns (Chiralcel OD-RH and Chiralpak AD-RH) with mobile phase consisting of a mixture of ammonium acetate buffer (pH 6.8) with acetonitrile or with potassium dihydrogen phosphate buffer (pH 4.5) and acetonitrile in various ratios. No separation was achieved in reversed phase chiral stationary phases.

The enantiomers were found to be separated only on Cellulose tris(3,5-dimethylphenyl)carbamate coated on silica gel based CSP (Chiralcel OD-H), with mobile phase comprising either a mixture of n-hexane, ethanol and TFA or n-hexane, 2-propanol and TFA. Owing to the smaller particle size of the stationary phase, the chiralcel OD-H yielded a better separation. In the case of CSPs with carbamate derivatives, binding of solute to the CSPs was achieved through the interactions between the solutes and the polar carbamate groups on CSPs[30]. Solutes can bind to the carbamate groups on the CSPs forming transient diastereomers through hydrogen bonding to C=O and NH groups and also through dipole-dipole interaction to the C=O moiety. These could contribute to the interactions with the carbamate groups on CSP, resulting in separation. The aromatic ring on the solute

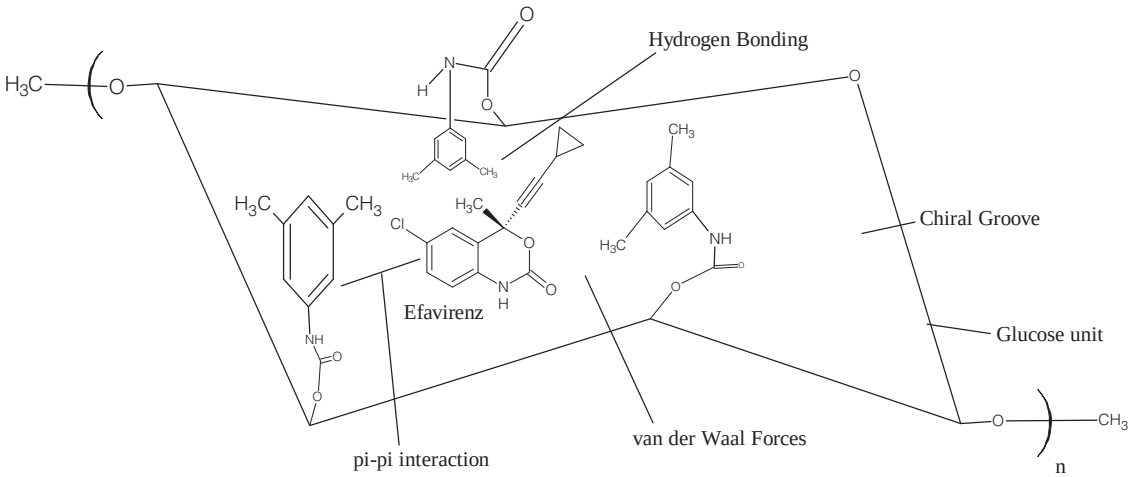

Figure 2. A representation of supra-molecular binding of EFZ on chiral groove of tris-(3, 5-dimethylphenyl) carbamate of cellulose unit (1, 4-connected-β-D-glucose).

could provide additional stabilizing effect to the solute-CSP complex by insertion of the aromatic portion of the solute into the chiral cavity[31]. In the present study, we too propose the existence of this type of stabilization effect due to the presence of aromatic functionality in EFZ. The representation of supra-molecular binding of EFZ on chiral groove of tris-(3, 5-dimethylphenyl) carbamate cellulose unit is represented in Figure 2.

The use of 2-propanol in the mobile phase provided better selectivity and resolution than ethanol. The addition of up to 0.05% (by volume) of TFA to the mobile phase resulted in improved peak shapes, better resolution and shorter run times.

TFA is the simplest stable perfluorinated carboxylic acid chemical compound. It is strong carboxylic acid due to the influence of the electronegative tifluoromethyl group. A majority of the enantioseparations of chiral acidic and basic compounds on the CSP require the addition of acidic mobile phase modifiers. TFA plays an important role in the adsorption-desorption process. Without the TFA modifier, EFZ enantiomers peaks exhibited tailing and poor separation and these were due to the strong interaction of EFZ with accessible silanol groups of the CSP. TFA essentially deactivated the CSP resulting in symmetrical peaks and better resolution. Furthermore, TFA displaced the analytes' acidic group on silanol sites of the CSP and suppressed the ionization of the weakly acidic silanol groups.

The effect of temperature, flow rate and concentrations of 2-propanol and TFA, on resolution (R_s) and retention time (t_R) were examined. The most optimum conditions were found to be a mobile phase consisting of n-hexane, 2-propanol and trifluoroacetic acid in the ratio of 90 : 10 : 0.05% (v/v/v) at a flow rate of 0.8 mL/min with the column maintained at ambient temperature. All the system suitability parameters including resolution, tailing factor and plate counts values were observed to be within the acceptable limits (Table 1). In the present method, the typical retention times of (S)-EFZ and (R)-EFZ were observed to be 7.5 and 9.2 min, respectively.

Table 1. System suitability parameters

Name	Retention Time (min)	USP tailing	Resolution	USP plate count
(S)- EFZ	7.556	1.326	Not applicable	7951
(R)- EFZ	9.223	1.359	2.921	3546

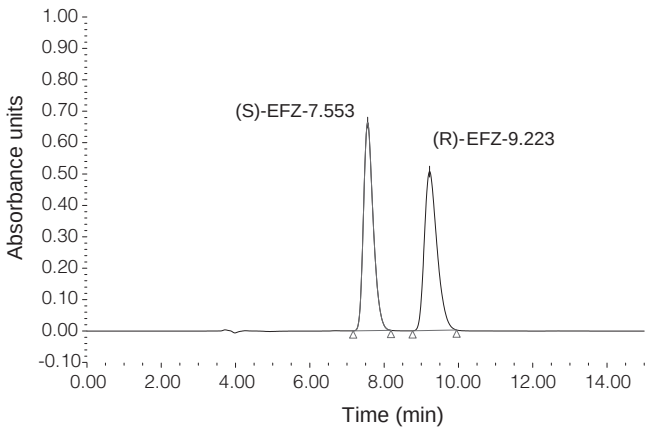

Figure 3. A typical chromatogram of EFZ (racemate) using an isocratic eluent of n-hexane: 2-propanol: TFA in 90 : 10 : 0.5% v/v for 15 min (UV absorption at 245 nm).

A typical chromatogram of the EFZ racemate is shown in Figure 3.

The specificity of the method was evaluated by analyzing the pharmaceutical excipients along with the dosage form (n = 5). The results were found to be in the range of 98.7 to 104.0% with RSD values of less than 1%. These results revealed the specificity of the proposed method.

II. *Limit of Detection (LOD) and Quantification (LOQ)*

The ICH guidelines were followed to calculate the LOD

and LOQ values. The LOD and LOQ were found to be 66 and 200 ng/mL, respectively. These results suggested that the proposed method is sufficiently sensitive for the determination of EFZ enantiomers.

III. *Precision*

The precision studies for (R)-EFZ were performed at LOQ level and at 1% of the analyte concentration. The % RSD values at LOQ level and 1% level were found to be 2.41 and 0.75, respectively. For inter-day and intra-day precision at 1% concentration, the % RSD values were noticed to be 1.39 and 0.54, respectively (Table 2).

IV. *Linearity and Range*

The calibration plot revealed a linearity range of 200 - 6210 ng/mL for (R)-EFZ with a determination coefficient value of 0.9999.

V. *Accuracy*

The recoveries were calculated from the slope and the intercept of the calibration plot for the (R)-EFZ standard. The recoveries ranged from 98.7 to 104.0% (Table 3). Large percentage recovery values revealed that the proposed method is accurate and could be adopted for routine quality-control analysis. A typical chromatogram of the spiked standard is shown in Figure 4.

VI. *Robustness*

In order to anticipate any problems that may arise during the application of the method, the method parameters, namely, flow rate, column temperature and percentage of trifluoroacetic acid in the mobile phase were intentionally varied and the effects were studied. The chromatographic resolution of the (S)-EFZ and (R)-EFZ peaks was used to

Table 2. Precision for (R)-EFZ

	% RSD
Repeatability at LOQ level [a]	
Retention time	0.03
Peak area	2.41
Repeatability at 1% level [a]	
Retention time	0.01
Peak area	0.75
Intra-day precision at 1% concentration [a]	
Retention time	0.02
Peak area	0.54
Inter-day precision at 1% level [a]	
Retention time	0.43
Peak area	1.39

[a] n = 6 determinations.

Table 3. Accuracy data for (R)-EFZ

Amount spiked (µg/mL)	Amount recovered (µg/mL)	Percentage Recovery
0.25	0.26 ± 0.009	104.0
3.19	3.15 ± 0.03	98.7
4.05	4.01 ± 0.07	99.0
4.96	4.99 ± 0.02	100.6

n=3

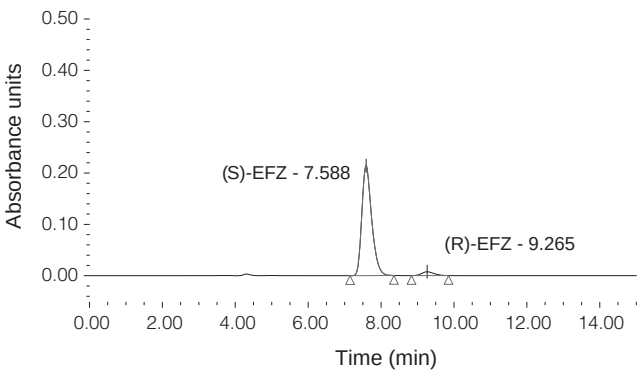

Figure 4. A typical chromatogram of (S)-EZ spiked with 1% (w/w) (R)-EFZ.

Table 4. Robustness of the chiral LC method

Parameter	USP resolution between (S)-EFZ and (R)-EFZ	% RSD
Flow rate (mL/min)		
0.6	3.0	9.4
0.8	2.9	
1.0	2.5	
Column temperature (°C)		
20	3.1	6.9
25	2.9	
30	2.7	
2-propanol percentage in mobile phase		
9	3.0	5.3
10	2.9	
11	2.7	
Trifluoroacetic acid percentage in mobile phase		
0.04	3.0	2.0
0.05	2.9	
0.06	2.9	

n=3

evaluate the robustness of the method under modified conditions. Sufficient resolution for (S)-EFZ and (R)-EFZ was obtained under all the tested separation conditions, thereby demonstrating sufficient robustness of the developed method (Table 4). The % RSD values for resolution between (S)-EFZ and (R)-EFZ were found to be less than 10 in the altered chromatographic conditions. Hence, it was concluded that the proposed method had good robustness for the analysis of EFZ in bulk sample or tablets.

VII. *Solution Stability*

No change in either the chemical or enantiomeric purity was observed. The peak areas for each isomer after 24 h did not show any significant change compared to those in the initial analysis step (Table 5). This indicated that both the isomers were stable in the mobile phase for at least 24 h when stored, either between 2 - 8°C or at bench top. A typical chromatogram of the (R)-EFZ standard is shown in Figure 5.

VIII. *Chiral Analysis of Bulk Drug and Formulations*

Various batches of bulk drug samples and pharmaceutical formulations were analyzed in triplicate. The results showed consistency between determinations with low % RSD values as shown in Table 6.

Table 5. Solution stability and mobile phase stability results of EFZ-Chiral LC method

Interval (h)	% (R)-EFZ (solution stability)	% (R)-EFZ (mobile phase stability)
Initial	0.15	0.15
6	0.16	0.14
12	0.15	0.14
18	0.14	0.15
24	0.15	0.15

Table 6. Analysis of EFZ in pharmaceutical formulations

Formulation	Labeled (mg)	Found*(mg)	% RSD	% Recovery*
SUSTIVA®	5	5.06	0.61	101.2
	10	9.91	0.69	99.1
	15	15.03	0.54	100.2
EFAVIR®	5	5.09	0.86	101.8
	10	9.96	0.71	99.6
	15	15.06	0.64	100.4

*Average of nine determinations.

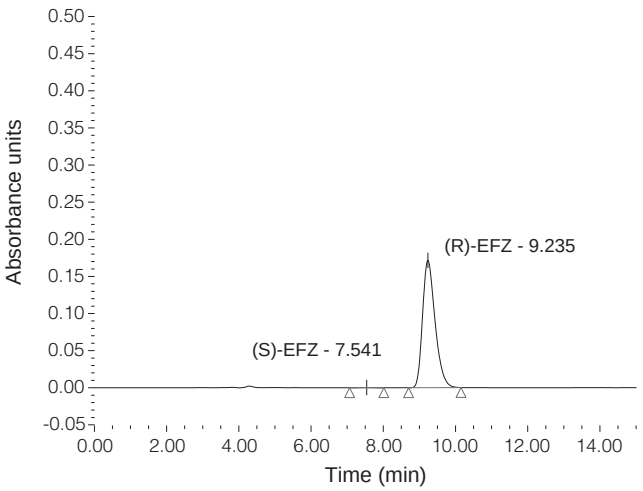

Figure 5. A typical chromatogram of (R)-EFZ standard (chemical purity: 99.85%; chiral purity: 99.51%).

CONCLUSIONS

A chiral HPLC method for the separation of EFZ enantiomers was developed and validated. The chiral separation was achieved using a cellulose carbamate derivatized column (Chiralcel OD-H). The proposed method is simple, accurate and precise. The practical applicability of this method was tested by analyzing various batches of the bulk drug sample and formulations. ICH guidelines were followed throughout the study for method validation. In view of this, the proposed method could be adopted for quality control and routine analysis.

ACKNOWLEDGMENTS

We are thankful to the authorities of the Mangalore University, Mangalagangotri and Karnatak University, Dharwad, for providing the necessary facilities.

REFERENCES

1. FDA's policy statement for the development of new stereoisomeric drugs. 1992. Chirality. 4: 338-340.
2. Aboul-Enein, H. Y. and Ali, I. 2003. Chiral Separation by Liquid Chromatography and Related Technologies. Marcel Dekker Inc, New York.
3. Ariens, E. J. 1986. Stereochemistry: a source of problems in medicinal chemistry. Med. Res. Rev. 6: 451-466.
4. Ariens, E. J. 1984. Stereochemistry, a basis for sophisticated nonsense in pharmacokinetics and clinical pharmacology. Eur. J. Clin. Pharmacol. 26: 663-668.
5. Ariens, E. J. and Wuins, E. W. 1987. Bias in pharmacokinetics and clinical pharmacology. Clin. Pharmacol. Ther. 42: 361-363.
6. Young, S. D., Britcher, S. F. and Tran, L. O. *et al*. 1995. L-743, 726 (DMP-266): A novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 39: 2602-2605.

7. Bartlett, J., Demasi, R., Quinn, J., Moxham, C. and Rousseau, F. 2000. Title: Proceedings of the 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, USA. (Abstract 519).
8. Gazzard, B. G. 2000. What are the perceived advantages and disadvantages of non-nucleoside reverse transcriptase inhibitors as first-line therapy. HIV Med. 1: 11-14.
9. Havlir, D., Hicks, C. and Kahn, J. *et al*. 1998. Proceedings of the 38th Inter-science Conference on Antimicrobial Agents and Chemotherapy, San Diego, USA. (Poster 1-104).
10. Villani, P., Pregnolato, M., Banfo, S., Rettani, M., Burroni, D., Seminari, E., Maserati, R. and Regazzi, M. B. 1999. High-performance liquid chromatography method for analyzing the antiretroviral agent efavirenz (EFV, DMP 266) in human plasma. Ther. Drug Monit. 21: 346-350.
11. Veldkamp, A. I., van Heeswijk, R. P., Meenhorst, P. L., Mulder, J. W., Lange, J. M., Beijnen, J. H. and Hoetelmans, R. M. 1999. Quantitative determination of efavirenz (DMP 266), a novel non-nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. B Biomed. Sci. Appl. 734: 55-61.
12. Langmann, P., Schirmer, D., Vath, T., Zilly, M. and Klinker, H. 2001. High-performance liquid chromatographic method for the determination of HIV-1 non-nucleoside reverse transcriptase inhibitor efavirenz in plasma of patients during highly active antiretroviral therapy. J. Chromatogr. B Biomed. Sci. Appl. 755: 151-156.
13. Sarasa-Nacenta, M., Lopez-Pua, Y., Lipez-Cortes, L. F., Mallolas, J., Gatell, J. M. and Carne, X. 2001. Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. B Biomed. Sci. Appl. 763: 53-59.
14. Matthews, C. Z., Woolf, E. J., Mazenko, R. S., Haddix-Wiener, H., Chavez-Eng, C. M., Constanzer, M. L., Doss, G. A. and Matuszewski, B. K. 2002. Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection. J. Pharm. Biomed. Anal. 28: 925-934.
15. Aymard, G., Legrand, M., Trichereau, N. and Diquet, B. 2000. Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. 744: 227-240.
16. Titier, K., Lagrange, F., Pehourcq, F., Edno-Mcheik, L., Moore, N. and Molimard, M. 2002. High-performance liquid chromatographic method for the simultaneous determination of the six HIV-protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in human plasma. Ther. Drug Monit. 24: 417-424.
17. Rezk, N. L., Tidwell, R. R. and Kashuba, A. D. M. 2002. Simple and rapid quantification of the non-nucleoside reverse transcriptase inhibitors nevirapine, delavirdine, and efavirenz in human blood plasma using high-performance liquid chromatography with ultraviolet absorbance detection. J. Chromatogr. B 774: 79-88.
18. Boffito, M., Tija, J., Reynolds, H. E., Hoggard, P. G., Bonora, S., Di Perri, G. and Back, D. J. 2002. Simultaneous determination of rifampicin and efavirenz in plasma. Ther. Drug Monit. 24: 670-674.
19. Cociglio, M., Hillaire-Buys, D., Peyriere, H. and Alric, R. 2003. Performance analysis of a rapid HPLC determination with the solvent demixing extraction of HIV antiproteases and efavirenz in plasma. J. Chromatogr. Sci. 41: 80-86.
20. Usami, Y., Oki, T., Nakai, M., Sagisaka, M. and Kaneda, T. 2003. A simple HPLC method for simultaneous determination of lopinavir, ritonavir and efavirenz. Chem. Pharm. Bull. (Tokyo) 51: 715-718.
21. Kappelhoff, B. S., Rosing, H., Huitema, A. D. and Beijnen, J. H. 2003. Simple and rapid method for the simultaneous determination of the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma using liquid chromatography. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 792: 353-362.
22. Keil, K., Frerichs, V. A., DiFrancesco, R. and Morse, G. 2003. Reverse phase high-performance liquid chromatography method for the analysis of amprenavir, efavirenz, indinavir, lopinavir, nelfinavir and its active metabolite (M8), ritonavir, and saquinavir in heparinized human plasma. Ther. Drug Monit. 25: 340-346.
23. Dailly, E., Raffi, F. and Jolliet, P. 2004. Determination of atazanavir and other antiretroviral drugs (indinavir, amprenavir, nelfinavir and its active metabolite M8, saquinavir, ritonavir, lopinavir, nevirapine and efavirenz) plasma levels by high performance liquid chromatography with UV detection. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 813: 353-358.
24. Poirier, J. M., Robidou, P. and Jaillon, P. 2005. Simple and simultaneous determination of the HIV-protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir plus M8 nelfinavir metabolite and the nonnucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma by reversed-phase liquid chromatography. Ther. Drug Monit. 27: 186-192.
25. Takahashi, M., Yoshida, M., Oki, T., Okumura, N., Suzuki, T. and Kaneda, T. 2005. Conventional HPLC method used for simultaneous determination of the seven HIV protease inhibitors and nonnucleoside reverse transcription inhibitor efavirenz in human plasma. Biol. Pharm. Bull. 28: 1286-1290.
26. Chenevier, P., Massias, L., Gueylard, D. and Farinotti, R. 1998. Determination of ethambutol in plasma by high performance liquid chromatography after pre-column derivatization. J. Chromatogr. B Biomed. Sci. Appl. 708: 310-315.
27. Investigational drug brochure: DMP 266. 1997. The

DuPont Merck Pharmaceutical Company. Wilmington, DE, USA.

28. Reddy, Y. S., Gotzkowsky, S. K., Eron, J. J., Kim, J. Y., Fiske, W. D., Fiscus, S. A., Petch, L., Cohen, M. S. and Kashuba, A. D. M. 2002. Pharmacokinetic and pharmacodynamic investigation of efavirenz in the semen and blood of human immunodeficiency virus type 1-infected men. J. Infect. Dis. 186: 1339-1343.
29. ICH Draft Guidelines on Validation of Analytical Procedures: Definitions and Terminology. 1995. Federal Register IFPMA, Switzerland. 60: 11260.
30. Yashima, E. and Okamoto, Y. 1995. Chiral discrimination oil polysaccharides derivatives. Bull. Chem. Soc. Jpn 68: 3289-3307.
31. Wainer, I. W., Stiffin, R. M., Shibata, T. 1987. Resolution of enantiomeric aromatic alcohols on a cellulose tribenzoate high-performance liquid chromatography chiral stationary phase: a proposed chiral recognition mechanism. J. Chromatogr. A 441: 139-151.