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Development and Validation of RP-Chiral HPLC Method for Determination of (*R*)-Enantiomer Excess Content in Efavirenz

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A simple, specific, linear, accurate and precise reverse phase chiral HPLC method was developed for the separation of efavirenz enantiomers by using the Lux Amylose-2 column containing amylose *tris*(5-chloro-2-methyl phenyl carbamate) as a stationary phase. The mobile phase consists of 0.1 % formic acid in water and acetonitrile (55:45, v/v). The flow rate was kept at 1.0 mL/min and the detection wavelength used 252 nm and the column temperature was set at 25 ºC. The limit of detection was 0.01 mg/mL and the limit of quantification was 0.04 mg/mL. The linearity calibration curve of (*R*)-enantiomer was shown well from the range of 0.04 mg/mL to 0.4 mg/mL. The values of the correlation coefficient were 0.999 and 0.999 for (*R*)-enantiomer and (*S*)-efavirenz, respectively. The percentage recoveries of (*R*)-enantiomer from efavirenz drug substance were ranged from 93.5% to 107.5%. The results demonstrated that developed RP-chiral HPLC method was simple, precise, robust and applicable for the estimation of (*R*)-enantiomer in efavirenz API. This method was validated in as per ICH Q2 (R1) and USP validation of compendial methods <1225>.

Keywords: Efavirenz, (*R*)-Enantiomer, RP-chiral HPLC, Validation.

INTRODUCTION

Efavirenz is chemically (4*S*)-6-chloro-4-(2-cyclopropyl-ethynyl)-4-(trifluoromethyl)-1*H*-3,1-benzoxazin-2-one and it's molecular formula is $C_{14}H_9NO_2ClF_3$ with molecular weight 315.675. Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTIs) with activity against HIV and one of the first AIDS drugs approved for once-daily dosing and most marketed pharmaceutical. It is used in combination with other antiretrovirals for the therapy of HIV [1-6]. The drug molecule contains one chiral center and the configuration of molecule is "*S*" (Fig. 1).

Most of the drugs exist as enantiomers, as the chemical property of enantiomers is different in a chiral environment, only one isomer is essential and having a positive effect while another enantiomer may lead unwanted side effects on human body [7-10]. According to the International Conference on Harmonization (ICH) guidelines, The United States Food and Drug Administration (US FDA) and other regulatory agencies, chiral identity, enantiomeric impurity and chiral assay tests are required for product specifications and quantify each enantiomer of the chiral drug individually [11,12].

Hence, it is important to encourage all the chiral drug molecules have to be separated and tested to remove the undesired isomer from the preparation and as well as to find optimal treatment and the right therapeutic control for the patient [13,14]. Chromatography on solid stationary phases is one of the most powerful separation techniques as a wide spectrum of possibilities offered by adjusting both the liquid mobile phase and the solid stationary phase to achieve the separation [15,16]. A literature survey reveals that few analytical methods have been established for determination efavirenz and its related substances in drug substance and estimation of (*R*)-enantiomer by normal phase chiral HPLC [17-24]. However, the separation of efavirenz enantiomers has not yet been carried out by RP-chiral HPLC. The more volatile solvents such as hexane, heptane, *etc*. in the normal phase methods can be avoided by developing the chiral separations by RP-Chiral HPLC.

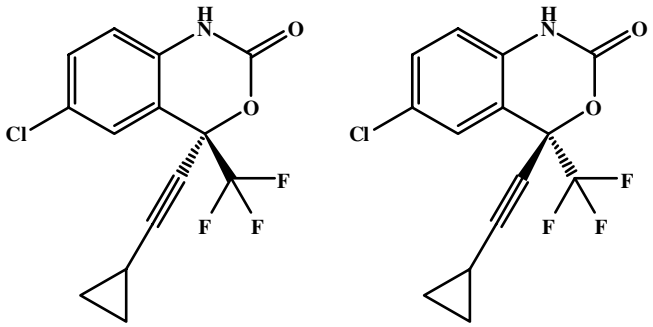

Fig. 1. Chemical structure of (*R*)-enantiomer and (*S*)-efavirenz

EXPERIMENTAL

Active pharmaceutical ingredient of efavirenz and efavirenz racemic mixture was synthesized by process research development of Laurus Labs Limited, Visakhapatnam. Formic acid (AR grade) and acetonitrile (HPLC grade) were purchased from the Sigma-Aldrich, India. High purity water was prepared using a Millipore Reference A^+ (Millipore, Milford, MA, USA) purification system.

Instrumentation: HPLC system with make Shimadzu LC-2010 CHT and weights measurements are taken by using an analytical balance with make Mettler Toledo XS205 dual range. Data were processed through LC solution software.

Chromatographic conditions: A column used is Lux Amylose-2, 250 mm × 4.6 mm, 5 µm particle size (Phenomenex) as stationary phase with a mobile phase 0.1% Formic acid in water and acetonitrile (55:45, v/v) at a flow rate of 1.0 mL min^{-1} and UV detection wavelength at 252 nm and 20 µL sample was injected for 15 min.

Preparation of system suitability solution: Weighed 10 mg of efavirenz racemic mixture standard and transferred into 100 mL volumetric flask, dissolved and diluted to volume with diluent and mixed well.

Preparation of standard solution: Weighed 50 mg of efavirenz standard and transferred into 100 mL volumetric flask, dissolved and diluted to volume with diluent and mixed well.

Preparation of mobile phase

Mobile phase-A: Pipetted 1.0 mL of formic acid into 1000 mL of Milli-Q water and mixed well, filtered through 0.45 µm filter paper and degassed.

Mobile phase-B: Used a filtered and degassed acetonitrile.

Diluent: Mixed 500 mL of water and 500 mL of acetonitrile, filtered through 0.45 µm filter paper and degassed.

RESULTS AND DISCUSSION

Method development and optimization: To develop the quantitative reverse-phase chiral HPLC method for (*R*)-enantiomer content in efavirenz, several trials were taken up by changing the columns, mobile phase and also different temperatures of the column. This HPLC method aims to get symmetry in the shape of the peak and to separate (*R*)-enantiomer and (*S*)-efavirenz with adequate resolution.

The attempts of method development were focused in reverse phases buffers such as ammonium acetate, formic acid, methanol, acetonitrile, *etc*. Initial method development attempts were made on different stationary phases such as cellulose *tris*(3,5-dimethylphenylcarbamate), cellulose *tris*(3-chloro-4-methylphenylcarbamate), cellulose *tris*-(4-methylbenzoate), cellulose *tris*(4-chloro-3-methylphenylcarbamate) and amylose *tris*(5-chloro-2-methylphenylcarbamate) on 5 µm silica-gel with different composition of solvent mixtures.

In this method development, the stationary phase has played a significant role in achieving the resolution between (*R*)-enantiomer and (*S*)-efavirenz. Finally, the satisfactory peak shape and resolution were achieved between two isomers on amylose *tris*(5-chloro-2-methylphenylcarbamate) column (250 × 4.6 mm and 5.0 µm particle size) by using formic acid solution and acetonitrile as mobile phase and a mixture of water and acetonitrile as diluent. These Lux polysaccharide-based chiral stationary phase columns was specially designed to resolve the stereoisomeric pharmaceutical compounds. Efavirenz molecule contains one chiral center and the absolute configuration of molecule is (*S*), hence the separation of isomers was effectively done by using the column of Lux Amylose-2. The method development trials are given in Table-1.

TABLE-1
RESULTS OF METHOD DEVELOPMENT

Trial	Column	Dimensions	Stationary phase	Mobile phase	Conclusion
1	Lux Cellulose-1	250 × 4.6 mm × 5.0 µ	Cellulose *tris*-(3,5-dimethylphenylcarbamate)	5 mM Ammonium acetate in water and acetonitrile	No separation
2	Lux Cellulose-2	250 × 4.6 mm × 5.0 µ	Cellulose *tris*-(3-chloro-4-methylphenylcarbamate)	5 mM Ammonium acetate in water and acetonitrile	No separation
3	Lux Cellulose-3	250 × 4.6 mm × 5.0 µ	Cellulose *tris*-(4-methylbenzoate)	5 mM Ammonium acetate in water and acetonitrile	No separation
4	Lux Cellulose-4	250 × 4.6 mm × 5.0 µ	Cellulose *tris*-(4-chloro-3-methylphenylcarbamate)	5 mM Ammonium acetate in water and acetonitrile	No separation
5	Lux Amylose-2	250 × 4.6 mm × 5.0 µ	Amylose *tris*-(5-chloro-2-methylphenylcarbamate)	5 mM Ammonium acetate in water and acetonitrile	No separation
6	Lux Amylose-2	250 × 4.6 mm × 5.0 µ	Amylose *tris*-(5-chloro-2-methylphenylcarbamate)	0.1 % Formic acid in water and methanol	Separation achieved but peaks are broad
7	Lux Amylose-2	250 × 4.6 mm × 5.0 µ	Amylose *tris*-(5-chloro-2-methylphenylcarbamate)	0.1 % Formic acid in water and acetonitrile	Separation achieved with sharp peaks

Method validation: The described (*R*)-enantiomer content in the efavirenz trial method was validated as per the ICH guidelines.

Specificity: The specificity of the method is performed by injecting the efavirenz system suitability solution (Fig. 2) and efavirenz standard solution (Fig. 3) individually. The specificity determined by using resolution between (*R*)-enantiomer and (*S*)-efavirenz. Baseline separation with a resolution of more than 3.0 achieved and the results are reported in Table-2.

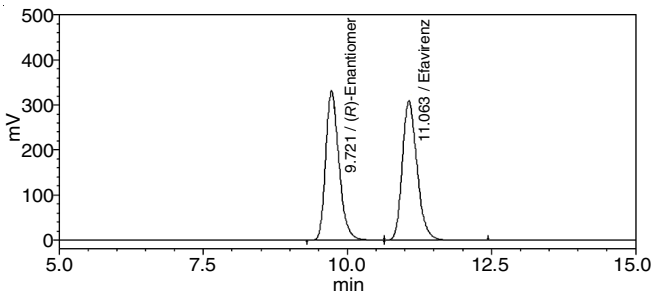

Fig. 2. System suitability chromatogram of (*R*)-enantiomer and (*S*)-efavirenz

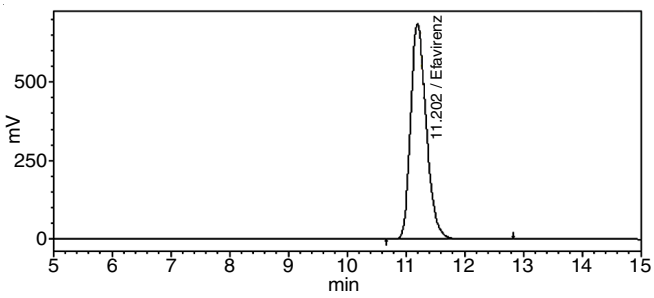

Fig. 3. Standard chromatogram of (*S*)-efavirenz

TABLE-2
SPECIFICITY RESULTS

Name	Retention time (min)	Plate count	Tailing factor	Resolution
(*R*)-Enantiomer	9.721	8652	1.3	–
(*S*)-Efavirenz	11.063	9018	1.3	3.04

Limit of detection (LOD) and quantification (LOQ): The LOD and LOQ for efavirenz and (*R*)-enantiomer were determined at a signal to noise ratio of 3:1 and 10:1, respectively, by injecting a series of a dilute solution with the known concentration of efavirenz and (*R*)-enantiomer. The precision study was carried out at the LOQ level by injecting six replicate injections individually and the % RSD for the area counts was calculated. The limit of detection and limit of quantification for (*R*)-enantiomer and efavirenz are reported in Table-3 and precision at LOQ in Table-4.

Precision: Method reproducibility was determined by measuring repeatability and intermediate precision of retention

TABLE-3
RESULTS LOD AND LOQ

Parameter	Conc. (mg/mL)	(*R*)-Enantiomer	(*S*)-Efavirenz	S/N ratio
LOD	0.01	1072	1295	17
LOQ	0.04	5197	4864	48

TABLE-4
RESULTS OF PRECISION AT LOQ

No. of preparation	(*R*)-Enantiomer	(*S*)-Efavirenz
1	5197	4865
2	4808	4837
3	4617	5132
4	5265	4854
5	5273	5228
6	4674	5160
Average	4972.3	5012.6
STDEV	306.2	178.9
%RSD	6.15	3.57

times and peak areas for enantiomer. The repeatability of the method was determined by analyzing six replicate injections containing efavirenz and (*R*)-enantiomer (each 0.2 mg/mL). The study was performed by the analyst on a different day, using different column and instrument and for two different days to study inter-day variation, and also prepare different solutions on different days. The % RSD of (*R*)-enantiomer found in the spiked samples was calculated.

The RSD (%) of peak area for (*R*)-enantiomer in the study of repeatability is shown in Table-5. Results for method precision (intra- and inter-day repeatability) are within 5.0%. These results confirm that the method is highly precise.

TABLE-5
PRECISION AT SPECIFICATION LEVEL

No. of preparation	(*R*)-Enantiomer area
1	26692
2	26247
3	27267
4	27603
5	27998
6	26759
Average	27094
STDEV	647.6
%RSD	2.4

Accuracy: The accuracy of the method was carried out by injecting a known concentration *i.e.* of (*R*)-enantiomer to efavirenz. The accuracy was calculated in terms of recovery (%) and the obtained chromatogram is shown in Fig. 4. It is observed that the percentage recovery of (*R*)-enantiomer in bulk drug samples ranged from 93.5% to 107.5% and results are reported in Table-6.

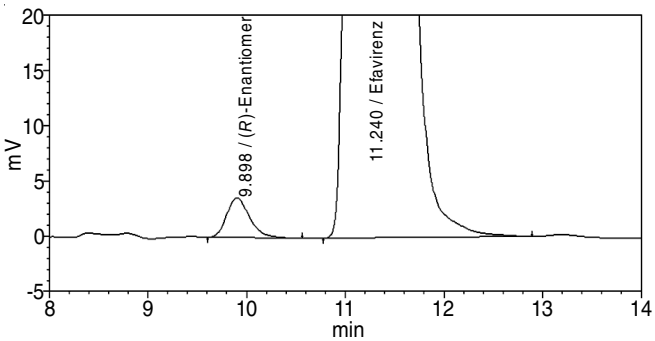

Fig. 4. Chromatogram of (*R*)-enantiomer at 0.2 mg/mL and (*S*)-efavirenz at test sample concentration

TABLE-6
RESULTS OF ACCURACY AT 0.2 mg/mL OF (*R*)-ENANTIOMER w.r.t SAMPLE CONCENTRATION

No. of preparation	Area (%)	Recovery (%)
1	0.187	93.5
2	0.215	107.5
3	0.208	104.0
4	0.196	98.0
5	0.200	100.0
6	0.209	104.5

Linearity : Detector response linearity was assessed by preparing eight calibration sample solutions of (*R*)-enantiomer covering from 0.04 to 0.4 mg/mL with respect to sample concentration in the diluent. The regression curve was obtained by plotting area *versus* concentrations using the least square method. The correlation coefficient, slope and Y-intercept of the calibration curve was calculated and the obtained plots are shown in Fig. 5. For (*R*)-enantiomer, linear calibration curve was obtained ranging from LOQ, 50, 80, 100, 120, 150 and 200%. The correlation coefficient obtained is greater than 0.999. The results indicate excellent linearity (Table-7).

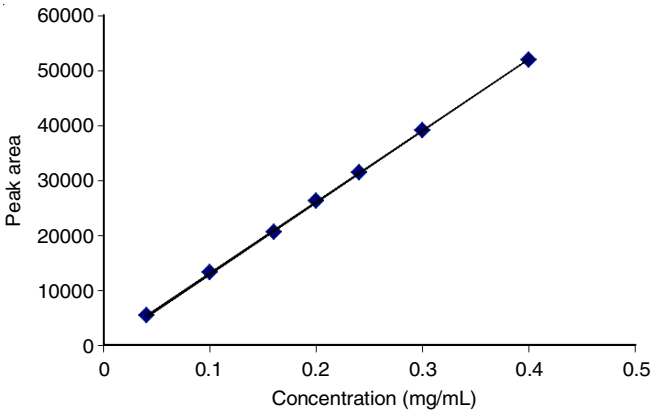

Fig. 5. Linearity plot for (*R*)-enantiomer

TABLE-7
LINEARITY OF (*R*)-ENANTIOMER

Level	Concentration (mg/mL)	Area
LOQ	0.04	5500
50	0.10	13291
80	0.16	20668
100	0.20	26247
120	0.24	31477
150	0.30	39193
200	0.40	51959
Correlation coefficient		0.999
Slope		129972
% Y-intercept		1.30

Robustness: To determine the robustness of method, the flow rate was changed from 0.9 to 1.1 mL/min. The effect of a change in the percent acetonitrile, column temperature at 20 and 30 ºC instead of 25 ºC were studied, and the other chromatographic conditions were held constant stated previously and obtained results of robustness are shown in Table-8.

Analytical solution stability: Solution stability was studied by keeping the test solution in the tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. Content of (*R*)-enantiomer was checked for every 12 h interval and compared with the freshly prepared solution. No variation was observed in the content of (*R*)-enantiomer for the study period and this indicates efavirenz sample solutions prepared in diluent were stable up to 48 h at room temperature.

TABLE-8
RESULTS OF ROBUSTNESS

Parameter		Resolution
Flow rate (mL/min)	0.9	2.99
	1.0	3.04
	1.1	3.01
Column temperature (°C)	20	3.11
	25	3.04
	30	2.96
Organic ratio	10	3.27
	15	3.04
	20	2.74

Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in efavirenz, which were prepared freshly at every 12 h interval for 48 h. The same mobile phase was used during the study period. No variation was observed in the content of (*R*)-enantiomer for the study period and it indicates prepared mobile phase was found to be stable up to 48 h at room temperature.

Conclusion

A simple, specific, linear, accurate and precise reverse phase chiral HPLC method was successfully developed, which was capable of separating the undesired enantiomer from efavirenz, column Lux Amylose-2 (250 mm × 4.6 mm, 5.0 µm) was found to be selective for enantiomers of efavirenz. The developed and validated method can be used for the estimation of (*R*)-enantiomer in efavirenz API. The developed method is also stable and can be used for the quantitative determination of chiral impurity in efavirenz drug substance form.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. M.M. Bastos, C.C.P. Costa, T.C. Bezerra, F. de C. da Silva and N. Boechat, *Eur. J. Med. Chem.*, **108**, 455 (2016); https://doi.org/10.1016/j.ejmech.2015.11.025
2. R. Gaida, I. Truter and C. Grobler, *South Afr. J. Psych.*, **21**, a783 (2015); https://doi.org/10.4102/sajpsychiatry.v21i3.783
3. B.M. Best and M. Goicoechea, *Expert Opin. Drug Metab. Toxicol.*, **4**, 965 (2008); https://doi.org/10.1517/17425255.4.7.965
4. J.C. Adkins and S. Noble, *Drugs*, **56**, 1055 (1998); https://doi.org/10.2165/00003495-199856060-00014
5. S.M.E. Vrouenraets, F.W.N.M. Wit, J. van Tongeren and J.M.A. Lange, *Expert Opin Pharmacother.*, **8**, 851 (2007); https://doi.org/10.1517/14656566.8.6.851
6. M. Maggiolo, *J. Antimicrob. Chemother.*, **64**, 910 (2009); https://doi.org/10.1093/jac/dkp334
7. A. Gogoi, N. Mazumder, S. Konwer, H. Ranawat, N.-T. Chen and G.-Y. Zhuo, *Molecules*, **24**, 1007 (2019); https://doi.org/10.3390/molecules24061007

8. L.A. Nguyen, H. He, C. Pham-Huy and C. Drugs, *Int. J. Biomed. Sci.*, **2**, 85 (2006).
9. N. Chhabra, M.L. Aseri and D. Padmanabhan, *Int. J. Appl. Basic Med. Res.*, **3**, 16 (2013); https://doi.org/10.4103/2229-516X.112233
10. S.W. Smith, *Toxicol. Sci.*, **110**, 4 (2009); https://doi.org/10.1093/toxsci/kfp097
11. A.G. Rauws and K. Groen, *Chirality*, **6**, 72 (1994); https://doi.org/10.1002/chir.530060206
12. S.K. Branch, *J. Pharm. Biomed. Anal.*, **38**, 798 (2005); https://doi.org/10.1016/j.jpba.2005.02.037
13. V. Famiglini and R. Silvestri, *Molecules*, **21**, 221 (2016); https://doi.org/10.3390/molecules21020221
14. A. Nag, Asymmetric Synthesis of Drugs and Natural Products, CRC Press: Boca Raton (2018).
15. B. Li and D.T. Haynie, ed.: S. Lee, Chiral Drug Separation, In: Encyclopedia of Chemical Processing, , CRC Press: Boca Raton (2005).
16. O. Coskun, *North. Clin. Istanb.*, **3**, 156 (2016); https://doi.org/10.14744/nci.2016.32757
17. K. Tachibana and A. Ohnishi, *J. Chromatogr. A*, **906**, 127 (2001); https://doi.org/10.1016/S0021-9673(00)00955-9
18. U. Seshachalam, D.V.L. Narasimha Rao and K.B. Chandrasekhar, *Pharmazie*, **63**, 107 (2008); https://doi.org/10.1691/ph.2008.7208
19. S.S. Pujeri, A.M.A. Khader and J. Seetharamappa, *J. Food Drug Anal.*, **21**, 93 (2013); https://doi.org/10.6227/jfda.2013210112
20. A.F. Aubry, D.S. Sebastian, R.C. Williams and R.J. Boucher, *Chirality*, **13**, 193 (2001); https://doi.org/10.1002/chir.1019
21. G. Lavison and D. Thiébaut, *Chirality*, **15**, 630 (2003); https://doi.org/10.1002/chir.10263
22. S.D. Sharma and G. Singh, *Adv. Anal. Chem.*, **3**, 29 (2013).
23. Efavirenz Monograph, USP42 NF37, p. 1554 (2019).
24. Efavirenz monograph, The International Pharmacopeia, edn 8 (2018).