BJPS

Brazilian Journal of
Pharmaceutical Sciences
vol. 47, n. 1, jan./mar., 2011

Article

Development and validation of a HPLC analytical assay method for efavirenz tablets: a medicine for HIV infections

Osnir de Sá Viana[1], Flávia Patrícia Morais Medeiros[2], Severino Grangeiro-Júnior[2], Miracy Muniz Albuquerque[1], Mônica Felts La Roca Soares[1-3], José Lamartine Soares-Sobrinho[1-3], Lariza Darlene Santos Alves[1], Larissa Araújo Rolim[1], Keyla Emanuelle Ramos da Silva[1], Pedro José Rolim-Neto[1*]

[1]Laboratory of Medicine Technology, Pharmaceutical Sciences Department, Pernambuco Federal University,
[2]Pernambuco State Pharmaceutical Laboratory – LAFEPE,
[3]Pharmaceutical Technology Nucleus, Department of Biochemistry and Pharmacology, Piauí Federal University

Efavirenz is a reverse transcriptase non analog nucleoside inhibitor used to treat HIV infections. A simple assay method by high performance liquid chromatography was developed and validated for efavirenz tablets. The physical chemical characteristics of efavirenz were investigated to developing the method. The method was validated observing the parameters described in USP 29. Analyses were performed by an ultraviolet detector at a 252 nm wavelength, on a reverse-phase column (C_{18}, 250 mm x 3.9 mm, 10 µm), using an isocratic mobile phase containing acetonitrile/water/orthophosphoric acid (70:30:0.1). The validation parameters used were: selectivity, linearity, precision, accuracy, robustness, detection and quantification limits, and all resulting data were treated by a statistical method. The results obtained confirmed an alternative assay method for efavirenz tablets adequate for routine industrial use.

Uniterms: Efavirenz/tablet assay. High performance liquid chromatography/quantitative analysis.

O efavirenz é um inibidor não análogo de nucleosídeo da transcriptase reversa, utilizado no tratamento da infecção por HIV. Um método simples, por cromatografia líquida de alta eficiência, foi desenvolvido e validado para quantificação do efavirenz em comprimidos. O desenvolvimento do método levou em consideração as características físico-químicas do efavirenz. O método foi validado seguindo os parâmetros da USP 29. A análise foi realizada por meio de detector ultravioleta, utilizando um comprimento de onda de 252 nm, com coluna de fase reversa (C_{18}, 250 mm x 3.9 mm, 10 µm) e fase móvel isocrática contendo acetonitrila/água/ácido ortofosfórico (70: 30: 0.1). Os critérios usados para validação foram: seletividade, linearidade, precisão, exatidão, robustez e limites de detecção e quantificação do método. Foi utilizado método estatístico em todas as etapas do processo de validação. Os resultados obtidos mostraram que o método é uma alternativa para quantificação do efavirenz em comprimidos, tornando viável seu uso na rotina industrial e laboratórios analíticos.

Unitermos: Efavirenz/determinação em comprimidos. Cromatografia líquida de alta eficiência/análise quantitativa.

INTRODUCTION

Efavirenz is an antiretroviral agent employed in the treatment of HIV infections. It is a non analog nucleoside antiretroviral and non competitive reverse transcriptase inhibitor. Efavirenz is directly connected to the enzyme and blocks RNA and DNA-dependent, DNA-polymerase activities, causing destruction of the enzyme catalytic site. It has a molecular form of $C_{14}H_9ClF_3NO_2$, and is optically active with a molecular weight of 315.68. This crystalline powder has a white or slightly yellowish appearance. The substance has a melting point ranging from 136.0 ºC to 141 ºC, is nearly water insoluble but is soluble in methanol and dichloromethane (Clercq, 2001).

Although several published analytic methods exist,

***Correspondence:** P. J. Rolim-Neto. Laboratório de Tecnologia dos Medicamentos, Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco. Av. Prof. Arthur de Sá, s/n - Cidade Universitária, 50740-521 - Recife – PE, Brazil. Phone/Fax: (+55 81) 3272-1383. E-mail: pedro.rolim@pq.cnpq.br

the majority seek to determine efavirenz by the HPLC technique associated with other antiretroviral drugs or their metabolites in biological fluids (Sarasa-Nacenta *et al*., 2001; Matthews *et al*., 2002).

For pharmaceutical forms, Montgomery and collaborators (2001) used reverse phase chromatography to analyze efavirenz and the impurities present in raw material and capsules. In the cited study, the authors used a cyan column and a gradient mobile phase, with different proportions of methanol, water, and trifluoroacetic acid, and a 40-min run time. However, we sought to develop a method in order to facilitate the pharmaceutical industry's quality control routine, and thus should combine fast analyses with reliable results.

The present study describes the development and validation of an analytical assay method for efavirenz raw material and tablets by HPCL. The isocratic method was used together with a reverse-phase C_{18} column, acetonitrile mobile phase and water acidified with orthophosphoric acid, and a 7 min run time, according to the parameters described in USP 29 (2006) and ICH (2005). The method developed offers advantages over other methods described in the literature.

MATERIAL AND METHODS

Equipment

The previously described chromatographic system comprised: Class VP (Shimadzu®) HPLC, Membrane Degasser, LC-10ADVP pumps, SIL-10ADVP self-injector, SPD-10 AVP detector, SCL-10 AVP controller, and CTO-10ASVP oven; C_{18} column (Lichcrocart®) 250 x 4 mm (10 µm) and C_8 column (Symmetry Waters®) 250 x 4.6 mm (5 µm).

Materials

The Efavirenz 600 mg coated tablet was developed in Pernambuco State Pharmaceutical Laboratory (LAFEPE), and has the following composition: efavirenz (Hetero Labs®, EF0230703); sodium sulfate lauryl (Nuclear®); hydroxypropylcellulose (Denver®); cellulose microcrystalline 250 and 101 (Blanver®); polyvinylpyrrolidone (Xiamem®); crospovidone (ISP Technologies®); croscarmellose (Rellance celulose®); magnesium esterase (Blanver®); colloidal silicon dioxide (Henkel®), opadry Y-1-7000 (Colorcon). The placebo used in the validation study was prepared with excipients only.

The following reagents were used: HPLC-grade acetonitrile (ACN) (JT Baker®), HPLC-grade methanol (JT Baker®), orthophosphoric acid (H_3PO_4) at 85% for analysis (Merck®). The purified water used was obtained using the reverse osmosis system (Milli-Q Millipore Corporation®).

Chromatographic conditions

The chromatographic conditions used to validate the dosage method for efavirenz, substance and finished product, were as follows: isocratic system with a mobile phase made up of ACN: water: 85% H_3PO_4 (70:30:0.1); flow of 1.0 $mL.min^{-1}$; oven temperature of 30 ºC; injection volume of 20 µL; $\lambda = 252$ nm; a C_{18} 250 x 4 mm (10 µm) column. The asymmetry factor (As) was calculated as 10% of the height of the chromatographic band (Snyder *et al.,* 1997).

Standard solution preparation

Efavirenz supplied by Hetero Labs (lot WS.EF0202, with 99.93% purity), substance chemical reference (SQR), was used for standard solution preparation. The efavirenz standard stock solution was prepared in a mobile phase ACN/water/85% H_3PO_4 (70:30:0.1), at a concentration of 400 $\mu g.mL^{-1}$. Dilutions with mobile phase were performed in order to obtain solutions with concentrations between 10 and 40 $\mu g.mL^{-1}$, where an average concentration of 20 $\mu g.mL^{-1}$ was defined as 100%.

Analytical method development

Different columns, mobile phase, flow, and column temperatures were tested in the development of the analytical method. C_8 and C_{18} columns of the same length and diameter were also tested, keeping the same parameters and conditions (1 $mL.min^{-1}$ flow, mobile phase, injection volume of 20 µL, temperature 30 ºC). For the mobile phase, methanol/water, ACN/water and ACN/water/85% H_3PO_4 mixtures were tested, with the other parameters kept constant. The mobile phase holdup time, resolution, efavirenz peak asymmetry, and quantity of fractions defined by the reading of area integrations from the chromatograms were assessed. The concentration of tested samples was 20 $\mu g.mL^{-1}$ throughout method development.

Validation study

For the process of defining the performance of the chromatographic system used, the following parameters were assessed: robustness, linearity, variation range, precision, accuracy, selectivity, detection and quantification limits for raw material and tablets.

Robustness

The method's robustness represented a measure of its ability to resist change in response to minor and deliberate variations in analytical parameters (ICH, 2005). Robustness was determined based on temperature variations, mobile phase proportion, flow, and different acetonitrile manufacturers (robustness for finished product assessed). The analyses were performed in sextuplicate.

Linearity

Linearity denotes the ability of the method to provide results directly proportional to the concentration of substance in question within a given application range (Swartz, Krull, 1998; ICH, 2005; USP, 2006). The method linearity was assessed by linear regression analysis using the least-squares methods for the average points of three authentic calibration curves, at concentrations of 10, 15, 20, 25, 30 and 40 $\mu g.mL^{-1}$. The variation range tested was 50-150% of analysis concentration. Linear model adjustment and regression validity tests were performed in order to check regression equation significance.

Precision

The method precision was assessed on two levels: repeatability and intermediate precision. The method was tested for these two precision levels based on 6 determinations (ICH, 2005) at 20 $\mu g.mL^{-1}$ concentrations. Intermediate precision was determined on different days by different analysts. Repeatability was expressed by the coefficient of variation (CV), whereas intermediate precision was expressed as CV and average reliance interval by *Student's t* test (Ribani *et al*., 2004).

Accuracy

The method accuracy was assessed at three concentration levels: 40, 100 and 160% for raw material, where 100% corresponded to 20 $\mu g.mL^{-1}$. In tablets, the evaluation was performed at concentrations of 70, 100 and 130%, which were incorporated into a given amount of placebo. The tests for both the raw material and tablets were conducted in sextuplicate for each concentration level and were assessed using *Student's* t test by comparing results obtained against theoretical values defined for each analyzed concentration.

Selectivity

To determine selectivity, the analyte was compared to placebo solution, verifying that no interference exists between the formulation excipients in the analysis method (Swartz, Krull, 1998; Ribani *et al*., 2004).

RESULTS AND DISCUSSION

Analytical method development

Despite presenting similar characteristics and behavior, a significant difference in the mobile phase holdup time using the C_8 and C_{18}, columns was observed, being shorter in the latter. On the tests with a mobile phase, no significant difference was observed between the results obtained with the mobile phase acidified with orthophosphoric acid and the mobile phase without acidification. However, the acidified mobile phase provides for better separation of probable synthesis impurities from the efavirenz, reducing the risks of interaction with other peaks.

The mobile phase made up of methanol and water at a (60:40) proportion produced efavirenz precipitation inside the column, although good results were obtained at 70:30 and 80:20 proportions. The mobile phase chosen for analytical method validation consisted of ACN/ water/ 85% H_3PO_4 (70:30:0.1), presented a mobile phase holdup time of 5.08 min, ease of manipulation, with good resolution and peak definition in the chromatogram. Tests were performed using the mobile phase and column defined, with the efavirenz being submitted to temperatures of 40 ºC and 80 ºC to assess the analyte behavior under these conditions. There was no variation in the integration of peak areas obtained at these temperatures, where a reduction occurred only within the mobile phase holdup time, due to the reduced viscosity of the mobile phase (Table I).

Analytical method validation

Linearity

The linear regression equation obtained by the proposed method using three authentic calibration curves was, $y = 56456.61x + 245.033$, where **y** represents the integrated peak area in the chromatogram, and **x** represents efavirenz concentration in $\mu g.mL^{-1}$.

The correlation coefficient obtained of 0.99997 demonstrates the good quality of the calibration curve, as the lower the dispersion of the set of points, the lower the uncertainty of the estimated regression coefficients. Using variance analysis, the model validation and the statistical significance of the adjusted curve can be tested. The data variance analysis demonstrated that the method is linear within the tested concentration range (10-40 $\mu g.mL^{-1}$), and that there is no lack of model adjustment, as the calculated F (0.92) is lower than the listed F (3.26), evidencing that the assumption of lack of adjustment is false.

TABLE I - Composition definition of the mobile phase and column for validation

Column	Mobile Phase	%	t_R (min)	Area	A_S
C_{18}	ACN/ H_20	60:40	8.56	1112004	1.26
C_{18}	ACN/ H_20	70:30	5.18	1110985	1.33
C_8	ACN/ H_20	70:30	7.24	1104365	1.28
C_{18}	ACN/ H_20	80:20	3.68	1096213	1.24
C_{18}	ACN/ H_20/85% H_3PO_4	60:40:0.1	8.52	1109042	1.18
C_{18}	**ACN/ H_20/ 85% H_3PO_4**	**70:30:0.1**	**5.08**	1128041	**1.15**
C_{18}	ACN/ H_20/ 85% H_3PO_4	80:20:0.1	3.70	1114729	1.19
C_{18}	MeOH/H_20	60:40	low solubility	-	-
C_{18}	MeOH/H_20	70:30	4.85	1107448	1.23
C_{18}	MeOH/H_20	80:20	3.77	1110316	1.31
Study of increased temperature in the chromatogram resolution					
Column	Mobile Phase	%	t_R (min)	Area	A_S
C_{18}	ACN/H_20/ 85% H_3PO_4 at 40 ºC	70:30:0.1	3.74	1119299	1.23
C_{18}	ACN/H_20/ 85% H_3PO_4 at 80 ºC	70:30:0.1	4.53	1118568	1.28

Robustness

The results for temperature robustness, mobile phase proportion, and acetonitrile supplier for the finished product dosage method, were statistically treated and are described in Table II. The method proved to be robust for the variations in the column temperature and mobile phase proportion assessed. The results of the variance analysis of Student's t test for assessed parameters showed no statistically significant difference between the variations in temperature and mobile phase proportion or the acetonitrile manufacturers assessed, with a 95% reliance interval.

Precision: Finished Product

- **Repeatability:** Method repeatability can be observed with a standard sampling deviation of 0.05% and a variation coefficient of 0.25%.
- **Intermediate Precision:** The intermediate precision results for the finished product showed the variation coefficient to be lower than 2% in all instances . On the significance tests assessing the method precision test results for the coated tablets analyzed, no statistically significant differences among average values between days or between analysts was found. The calculated t was 0.53; 1.80; 2.04 and 0.16 for between analysts day 1; between analysts day 2; between days analyst 1 and between days analyst 2, respectively, with all values lower than the critical t (2.23).

Accuracy

The results of the method accuracy study yielded a CV of 1.4; 0.61 and 0.53% for the theoretical concentrations of 70, 100 and 130%, respectively. A statistical significance analysis was conducted using Student's t test. The calculated t was 0.22; 1.04; 2.46 respectively, with all values lower than the critical t (2.57).

In Figure 1, an efavirenz chromatogram obtained under chromatographic conditions shows a single well-defined peak of efavirenz, with a 1.1 asymmetry. Based on the data observed from the method accuracy study, no statistically significant difference was found, with a 95%

TABLE II - Finished product statistical study

PARAMETERS	F cal.	P value	F critical	t cal.	t critical
Temperature (ºC)	0.14	0.87	3.68	-	-
Mobile phase (ACN/H_20 acidif.)	3.37	0.06	3.68	-	-
ACN Manufacturer	-	-	-	0.15	2.23

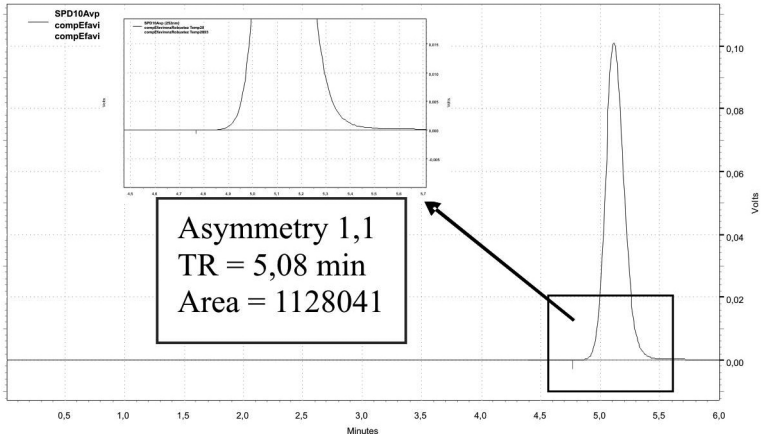

FIGURE 1 - Representative chromatogram obtained with the method precision

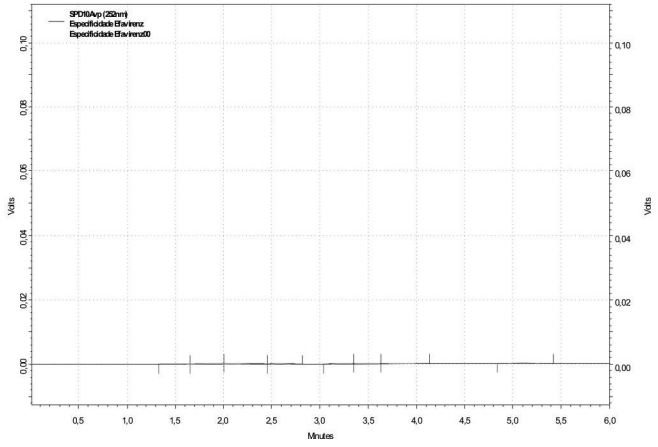

FIGURE 2 – Efavirenz Tablet chromatogram obtained for selectivity parameter

reliance interval, between the obtained results and the defined theoretical values, showing that the method for determination of the efavirenz in tablets is accurate.

Selectivity

Comparison of the chromatograms obtained for the placebo solution and efavirenz tablets revealed no significant interference of formulation excipients for the mobile phase holdup time of 5.08 min, using the same chromatographic conditions for both samples. Figure 2 depicts chromatograms of placebo and efavirenz tablets showing that the method is selective for the analyte concerned.

CONCLUSION

The method presented proved to be a straightforward alternative assay for efavirenz raw material and tablets, being robust, linear, precise, accurate, and selective, with well-defined peak reproduction and good resolution. The method developed therefore represents an alternative method in the laboratory routine of pharmaceutical industries, particularly those developing and producing anti-retroviral medications. The results obtained showed that the method complies with good laboratory practice requirements and meets the validation criteria set forth in USP and ICH guidelines.

ACKNOWLEDGMENTS

This study was supported by a grant from Pernambuco Federal University.

REFERENCES

CLERCQ, E. D. Antiviral drugs: current state of the art. *J. Clin. Virol.*, v.22, n.1, p.73-89, 2001.

ICH Q2 (R1). Validation of Analytical Procedures: Text and Methodology. Availabnle at: http://www.ich.org/LOB/media/MEDIA417.pdf. Accessed on: 20 mar. 2009.

MATTHEWS, C. Z.; WOOLF, E. J.; MAZENKO, R. S.; HADDIX-WIENER, H.; CHAVEZ-ENG, C. M.; CONSTANZER, M. L.; DOSS, G. A.; MATUSZEWSKI, B. K. 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.*, v.28, n.5, p.925-934, 2002.

MONTGOMERY, E. R.; EDMANSON, A. L.; COOK, S. C.; HOVSEPIAN, P. K. Development and validation of a reverse-phase HPLC method for analysis of efavirenz and its related substances in the drug substance and in a capsule formulation. *J. Pharm. Biomed. Anal.*, v.25, n.2, p.267-284, 2001.

RIBANI, M.; BOTTOLI, C. B. G.; COLLINS, C. H.; JARDIM, I. C. S. F. Validation in chromatographic and electrophoretic methods. *Quim. Nova*, v.27, n.5, p.771-780, 2004.

SARASA-NACENTA, M.; LÓPEZ-PÚA Y.; LÓPEZ-CORTÉS, L. F.; MALLOLAS, J.; GATELL, J. M.; CARNÉ, X. Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr., B: Biomed. Sci. Appl.*, v.763, n.1/2, p.53-59, 2001.

SNYDER, L. R.; KIRKLAND, J. J.; GLAJCH, J. L. *Practical HPLC method development*. 2.ed New York: Wiley, 1997. 765 p.

SWARTZ, M. E.; KRULL, I. S. Validação de métodos cromatográficos. *Pharm. Technol.*, v.2, n.3, p.12, 1998.

UNITED States Pharmacopoeia. 29.ed. Rockville: United States Pharmacopeial Convention, 2006. p.1567-1572.

Received for publication on 40th May 2009
Accepted for publication 25th August 2010