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

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INTRODUCTION

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 (C18, 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.


Efavirenz is a reverse transcriptase non analog nucleoside inhibitor used to treat 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 C14H9ClF3NO2, 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 °C to 141 °C, is nearly water insoluble but is soluble in methanol and dichloromethane (Clercq, 2001).

Although several published analytic methods exist,
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 (Lichrocart®) 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®); crosovidone (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$_3$PO$_4$) 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$_3$PO$_4$ (70:30:0.1); flow of 1.0 mL.min$^{-1}$; oven temperature of 30 ºC; injection volume of 20 µL; λ = 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$_3$PO$_4$ (70:30:0.1), at a concentration of 400 µg.mL$^{-1}$. Dilutions with mobile phase were performed in order to obtain solutions with concentrations between 10 and 40 µg.mL$^{-1}$, where an average concentration of 20 µg.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$_3$PO$_4$ 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 µg.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.
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RESULTS AND DISCUSSION

Analytical method development

Despite presenting similar characteristics and behavior, a significant difference in the mobile phase holdup time using the C₈ and C₁₈ 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% H₃PO₄ (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 \( \mu \text{g.mL}^{-1} \).

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 \( \mu \text{g.mL}^{-1} \)), 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.
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%
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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.

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