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Rapid and sensitive quantification of efavirenz in rat plasma using HPLC-MS/MS method

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This article aims to analyze the development of a rapid and sensitive HPLC–MS/MS method for quantification of efavirenz in rat plasma. The developed method included a liquid-liquid extraction process with methyl-*tert*-butyl ether solution, where hydrochlorothiazide was used as internal standard. The analyte was separated on an ACE Phenyl C18 column and eluted by a system consisting of mobile phase A (0.2% acetic acid) and mobile phase B (acetonitrile) at a proportion of 35:65 (*v/v*), pumped with a flow rate of 1.0 mL min^{-1} (run time < 4 min). Mass spectrometric detection was performed on a triple quadrupole instrument using multiple reaction monitoring. The electrospray ionization source was performed in negative ion mode. The precursor/product ion pairs monitored were m/z 313.644→68.800 and m/z 295.541→268.700 for efavirenz and internal standard, respectively. The limit of quantification was 10.0 ng mL^{-1} and calibration curves were linear over 10.0–1,500 ng mL^{-1}. Intra-day and inter-day precision at three levels were 1.80–10.49% and 4.72–10.28%, respectively. Accuracy ranged between 93.54% and 105.73%. Finally, the described method was applied to rats administered with efavirenz, demonstrating the suitability for quantification of efavirenz in a pharmacokinetic study. Therefore, it can be used in normal, hemolyzed or lipemic samples for efavirenz quantification.

Keywords: efavirenz; rat plasma; HPLC–MS/MS; pharmacokinetics

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Introduction

In the highly effective antiretroviral therapy protocol, the efavirenz, a drug commonly used in the treatment of human immunodeficiency virus type 1 infection, is a non-nucleoside reverse transcriptase (NRTI) inhibitor. This drug is used in combination with other antiretroviral agents that block the action and multiplication of HIV-1(1). Although antiretroviral therapy has contributed significantly to the improvement of the patient and management of the disease, its use also has several disadvantages and inconveniences for the patient. The serious associated side effects can be attributed to the subsequent high doses, which are essential to achieve a therapeutic effect due to inadequate concentrations of the drug at the site of action, and / or the low bioavailability of efavirenz, leading to formulation difficulties (2).

As a drug that is poorly soluble in water, belonging to class II of the biopharmaceutical classification system, efavirenz is a relevant case in the search for technological solutions that allow a more uniform and prominent dissolution and also an increase of bioavailability that guarantees relevant plasma levels (3). The lack of availability of adequate, easy-to-use and low-cost pediatric antiretroviral formulations is one of the greatest obstacles to adequate care for children with HIV. Several works can be identified that aim to increase the dissolution and bioavailability of efavirenz, but none has reached a stage of evolution that leads to the industrial development of a formulation and a product that can be supplied to the pediatric population. Strickley et al. (4) point out that most pediatric formulations available on the market are solutions and powders for suspension. The main objective of our research group is to find a system for increasing dissolution that can preferably allow the production of solid pediatric formulation orally, as recommended by the World Health Organization (WHO). Since the biggest problem is the low oral bioavailability of efavirenz, new technologies such as nanoparticles (5), microparticles (6), co-micronized systems (7), cyclodextrins (8) or solid dispersions (9) are being explored as an alternative to increase the bioavailability of the drug, allowing new therapeutic benefits for patients.

Monitoring the plasma level of efavirenz is essential, due to drug interaction and toxicity. Because of that; for the bioavailability to be assessed, it is necessary to have a sensitive, precise and accurate analytical methodology. The determination of this drug has already been conducted in different matrices such as hair strands (10-11), serum and urine (12), cervicovaginal fluid (13), mononuclear cells (14-16), and dried blood (17-19). Although analytical techniques such as capillary electrophoresis (20) have been used, it is unusual, since it presents a long period of analysis and extensive sample preparation, in addition to the use of micellar chromatography (21-22). Different methods using UV or LC-MS are described (23-32), having these applied to human plasma (33-42).

Since different substances could impact matrix effects, it is essential to test different sources of matrices during assay validation. The ionization of the analyte can be impacted by matrix effect, as well as extraction efficiency (43). It is known that there are differences between the human and animal plasma matrices and there is a lack of investigation of efavirenz in the plasma matrix of rats. Therefore, the objective of this work was to develop and validate an analytical methodology that presented a short period of analysis, for the quantification of efavirenz in a plasma matrix of rats, in order to support the future determination of nanotechnological formulations as enhancers of bioavailability for efavirenz.

Experimental section

Chemicals and reagents

Efavirenz (purity, 99.6%, Figure 1) and the internal standard (IS) hydrochlorothiazide (purity, 99.7%) were purchased from the Brazilian Pharmacopoeia (Brasília, DF, Brazil). HPLC-grade acetonitrile was purchased from Tedia (São Paulo, Brazil). Anesthetic ethyl carbamate was purchased from Sigma-Aldrich (Brazil). Methyl *tert*-butyl ether (MTBE) and HPLC-grade methanol were purchased from Scharlau (Barcelona, Spain). Acetic acid was purchased from Bio-Grade (CA-USA). Purified water was obtained by using Milli-Q system from the Millipore® (Bedford, MA, USA).

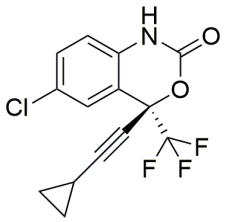

Figure. 1. Chemical structure of efavirenz.

Instrumentation and analytical conditions

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with an LC-A20AD$_{XR}$ pump, a DGU20A5R degasser, and a HTSPAL auto sampler (CTC Analytics) equipped with a thermostatic column oven (CTO-20AC). The chromatography was performed using Phenyl 5 µm RP-18 (150 mm × 4.6 mm) (ACE) at 40°C temperature. Efavirenz was eluted by a system consisting of mobile phase A (0.2% acetic acid) and mobile phase B (acetonitrile) at a proportion of 35:65, *v/v*. The mobile phase was pumped at a flow rate of 1.0 mL min^{-1} and the injection volume was 10 µL. Mass spectrometric detection was performed on an API 4500 triple quadrupole instrument (ABSCIEX, CA, USA) using multiple reaction monitoring (MRM). The electrospray ionization source was performed in negative ion mode. The precursor/product ion pairs monitored were m/z 313.644→68.800 for efavirenz and m/z 295.541→268.700 for IS, respectively. The turbo-gas temperature was set at 600 °C and the ion spray needle voltage was adjusted at -4500 V. The ion source gas 1, ion source gas 2, curtain gas and collision gas were set at 35, 50, 30, and media, respectively. Data acquisition was performed with Analyst software (Version 1.6.2).

Preparation of standards and sample extraction procedure

Before samples preparations, the efavirenz and IS were progressively diluted in methanol-water mixture (v/v, 4:6). Appropriate amounts of efavirenz and IS were dissolved in methanol to prepare separate stock solutions (0.2 mg mL^{-1}). The standard samples were prepared by adding an aliquot of 25 µL standard solution or quality control (low-LQC, medium-MQC and high-HQC) solutions and 25 µL of IS solution (3,000 ng mL^{-1}) to spike with 25 µL blank rat plasma to yield calibration standards in plasma in the concentration range of 10–1,500 ng mL^{-1} and HQC = 1,200 ng mL^{-1}, MQC = 600 ng mL^{-1} and LQC = 30 ng mL^{-1}. The extraction procedure was performed as follows: a volume of 25 µL blank rat plasma was added in the same volume of efavirenz and IS solution in a plastic tube. Then, 1.0 mL of the extraction solvent MTBE was added into the mixture and centrifuged at 14,400 rpm for 5 min at 10 °C. An aliquot of 0.75 mL of the upper layer was evaporated in nitrogen evaporator till dry. The dried extract was then reconstituted with 0.75 mL of mobile phase, vortex-mixed for 1 min and finally, 10 µL was injected into the LC-MS/MS system using the MRM.

Method validation

According to the FDA guidelines (44), the method was validated taking into consideration the following parameters: selectivity, linearity, lower limit of quantification, precision, accuracy, recovery, matrix effect, carryover, dilution integrity and stability.

Selectivity

Selectivity was determined by comparing six individual samples of blank rat plasma with the lowest point of the standard curve (LLOQC) for the appearance of co-eluting peaks/endogenous matrix components.

Linearity and lower limit of quantification

Calibration curves (n = 9) of efavirenz were constructed using eight concentrations levels: 10, 20, 50, 100, 300, 600, 1,200 and 1,500 ng mL^{-1}. The peak area ratios of efavirenz to IS *versus* the concentration data were treated by least-squares linear regression analysis (1/concentration^2). A weighting regression was employed for the linearity data. The intercept, slope, and coefficient of determination (r^2) were calculated. The acceptance criterium for the linearity was r^2> 0.99. Maximum deviation of standards was set at 15% of the stated value, excluding the LLOQC where deviation was set at no more than 20%.

Precision and accuracy

The accuracy and precision of the assay were determined by performing replicate analyses of quality control (QC) samples (LLOQC, LQC, MQC, HQC and DQC (dilution

quality control)) in six replicates and evaluated on three consecutive days. Intra-day and inter-day precision were analyzed. The accuracy and precision values should comply the requirements where intra-day and inter-day precision (RSD) should be < 15%, and accuracy must be within ± 15 %. DQC samples were prepared and diluted from a normal QC sample (1,200 ng mL^{-1}) employing a blank biological matrix to bring the analyte concentration within the calibration range.

Matrix effect

The matrix effect was assessed by the ratio of the efavirenz or IS in the post extraction blank biological matrices, comparing to the corresponding efavirenz or IS present solution. It was investigated matrix effect in normal, hemolyzed and lipemic plasma considering LQC and HQC. The results of the comparisons were expressed as percentages and must be within ± 15 %.

Carryover

Residual carryover was evaluated by checking the response following injection of a blank plasma immediately after extracted 1,500 ng mL^{-1} standard, followed by injections of the mobile phase. Efavirenz/IS carryover was evaluated as the percentage of peak area at is the drug retention time in standard blank to the same parameter in the upper limit of quantification standard.

Stability experiments

LQC and HQC were selected to investigate the stability experiments with eight replicates for each. Stabilities of efavirenz and IS were investigated including long-term stability (previously stored at -70 °C for 260 days, and kept at the room temperature for 5 hours before the analysis), auto-sampler stability (24h), six freeze-thaw cycles stability (-70 °C to room temperature) and short-term stability (5h). The stability result should be in the limits of 85–115%.

Application to a pharmacokinetic study

The validated bioanalytical method was applied to an in vivo study to determine efavirenz concentrations in a pharmacokinetic pilot study. Male Wistar rats (n = 4) (body weight: ~ 320 g) were employed. The experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and Institutional guidelines. The experimental protocols were authorized by the Animal Ethics Committee of University of Caxias do Sul (protocol # 006/2019). Water was provided *ad libitum* prior to the experiment. The rats were fasted overnight prior to dosing. The animals were anesthetized with ethyl carbamate (1.25 g kg^{-1}, i.p.) and received a single oral dose (20 mg kg^{-1}) of efavirenz formulation by oral gavage. This was a microcrystal formulation, as already detailed in Hoffmeister et al. (6). All details of the formulation can be found in this publication, including its physicochemical characterization and dissolution data. Briefly, a coarse suspension was prepared based on efavirenz, hydroxypropylmethylcellulose and sodium lauryl sulfate. This was processed in a colloidal mill and subsequently freeze-dried. The powder obtained did not demonstrate any change in the crystalline phase of efavirenz, with basically a change in particle size and wettability observed, parameters that were determined to be mandatory for increasing in vitro dissolution. The efavirenz suspension for gavage was made with carboxymethyl cellulose 1.0 %. The carotid artery was cannulated employing polyethylene tubing (.23"X.038"X.0075") and remained with 1% of heparinizing solution for blood collections. Blood samples were collected into heparinized tubes at pre-determined times (0.5, 1, 1.5, 2, 4, 6, 8 and 10 h) and then centrifuged at 12,000 rpm at 4 °C for 10 min and the supernatant plasma was stored -70 °C until quantification. The rats were euthanized with isoflurane at the end of the experiments.

Results

Method validation

Linearity

The calibration curve ranging from 10.0 ng mL^{-1} to 1,500 ng mL^{-1} fitted with 1/concentration^2 weighting factor was linear with coefficient of determination (r^2) > 0.998.

Selectivity

The retention time achieved for efavirenz and IS was about 3.20 and 1.88 min, respectively. Typical MRM chromatograms of efavirenz and IS are shown in Figure 2.

Carryover

It was observed that there was no contribution from the analyte and IS after a predetermined sequence of injections (Figure 2F). The result was considered satisfactory since no residual chromatographic signal was observed in these tests.

Matrix effect

It was observed through the calculation that the bioanalytical method did not present significant interferences in the recovery of efavirenz and IS from the extraction in plasma, both for LQC and HQC in normal, hemolyzed or lipemic matrices. The average of the LQC and HQC samples was 9.66% and 4.80%, respectively (RSD < 15%).

Precision and accuracy

Intra-day and inter-day precision and accuracy were evaluated for efavirenz at LLOQC, LQC, MQC and HQC levels. The dilution integrity was also evaluated as DQC. The results are presented in Table 1. The accuracies and precision successfully met the target acceptance criteria with RSD < 15% for the QC samples, except for the LLOQ which was < 20%. The results confirmed the acceptability of the dilution prior to sample analysis.

Stability experiments

Table 2 lists the results of short-term stability, post-preparative stability, long-term stability and freeze-thaw stability. The stock solutions of efavirenz and IS were stable with 1.57–3.91 % and 2.25–3.08% change from the initial concentration after 14 days at -70 °C condition, respectively, and 1.12–4.62% and 1.09–1.67 change after 23h of storage at room/ambient temperature.

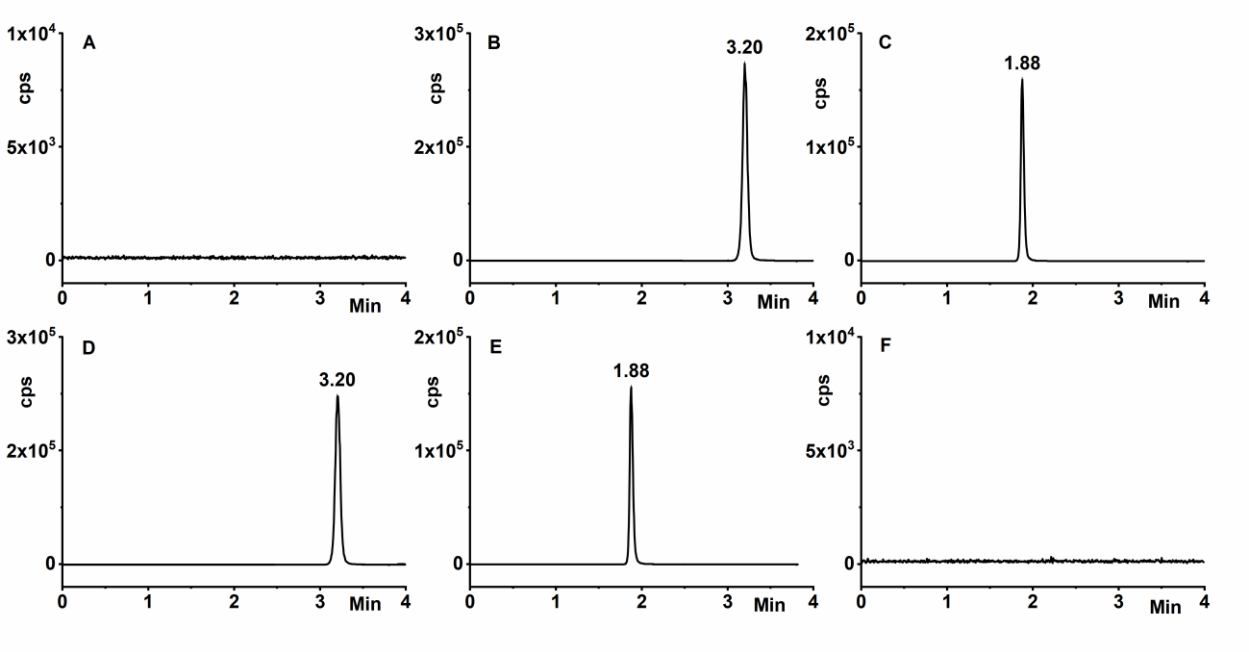

Figure 2. Representative selected reaction monitoring chromatograms in blank plasma sample (A), blank plasma sample spiked with efavirenz (B) and IS (C), plasma sample obtained from a rat after oral administration of efavirenz at 2 h (D-efavirenz, E-IS), and carry over (F).

Table 1. Intra- and inter-day precision and accuracy for quality control samples of efavirenz in rat plasma.

Nominal concentration	Day	Intra-day			Inter-day		
		Measured concentration			Measured concentration		
		Mean (ng mL^{-1})	Accuracy%	%RSD[a]	Mean (ng mL^{-1})	Accuracy%	%RSD[a]
LLOQ[b], 10 ng mL^{-1}	1	10.57	105.73	10.49			
	2	9.35	93.54	9.72	9.88	98.81	10.28
	3	9.71	97.12	6.75			
LQC[c], 30 ng mL^{-1}	1	30.71	102.37	5.16			
	2	30.89	102.98	6.79	30.46	101.53	5.44
	3	29.77	99.24	3.80			
MQC[d], 600 ng mL^{-1}	1	609.72	101.62	2.75			
	2	596.88	99.48	9.35	602.16	100.36	5.51
	3	599.94	99.99	1.80			
HQC[e], 1,200 ng mL^{-1}	1	1,242.36	103.53	6.10			
	2	1,264.44	105.37	2.52	1238.88	103.24	4.72
	3	1,209.84	100.82	4.33			
DQC[f], 1,200 ng mL^{-1}	1	1,127.04	93.92	6.78			
	2	1,253.52	104.46	3.99	1214.16	101.18	8.70
	3	1,214.16	101.18	9.66			

[a]%RSD = relative standard deviation; [b]LLOQ = lower limit of quantification; [c]LQC = low quality control; [d]MQC = medium quality control; [e]HQC = high quality control; [f]DQC = dilution quality control

Table 2. Autosampler stability, short-term stability, long-term stability and freeze-thaw stability of efavirenz at low and high quality control under specified conditions.

Efavirenz	Autosampler stability 4 °C, t = 12 h		Short-term stability room temperature t = 5h		Long-term stability −70 °C		Freeze-thaw stability, 3 cycles	
	Accuracy%	%RSD[a]	Accuracy%	%RSD[a]	Accuracy%	%RSD[a]	Accuracy%	%RSD[a]
LQC[b], 30 ng mL^{-1}	98.03	5.76	95.37	4.93	105.2	7.15	96.10	2.45
HQC[c], 1,200 ng mL^{-1}	96.43	4.01	102.47	3.00	91.8	4.53	98.10	1.49

[a]RSD = relative standard deviation; [b]LQC = low quality control; [c]HQC = high quality control.

Application to a pharmacokinetic study

The developed and validated LC-MS/MS method was applied to an *in vivo* study in Wistar rats treated with efavirenz (oral dosing). The mean plasma concentration-time profile of efavirenz is shown in Figure 3. The major pharmacokinetic parameters in this pilot study were: $t_{1/2}$ = 5.83 ± 0.82 h), T_{max} = 0.63 ± 0.25 h, C_{max} = 420.30 ± 96.14 ng mL^{-1}, $AUC_{0\text{-infinite}}$ = 2538.29 ± 572.19 h ng mL^{-1}.

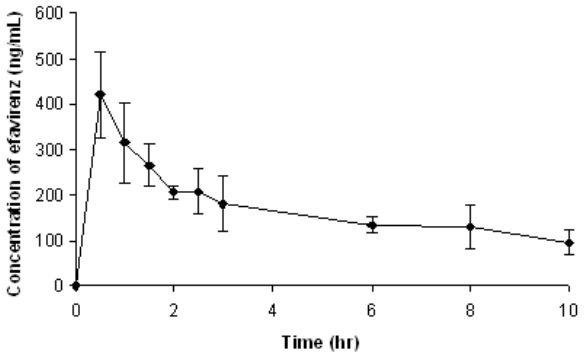

Figure 3. Mean concentration-time profile (mean ± SD) after 20 mg/kg oral dose of efavirenz in rat plasma (n = 4).

Discussion

Several methods have already been described for the quantification of efavirenz, although few in biological rodent matrix (35, 45-47). The developed and validated method here described represents the description of a quick analysis of efavirenz to be used in the investigation phase of formulations with technological changes in rodents. Initially, infusion tests were performed with the analyte and internal standard to define the fragmentation profile, considering the fragmentation energies and signal optimization. Subsequently, the temperature and gas conditions for sample drying were optimized and further optimization of analytical conditions such as flow and mobile phase was performed.

The efavirenz analyte can be ionized both positively and negatively, which is why both were investigated. However, a better profile was observed for negative ionization as present in other references (35, 45, 48). After the optimization of the analyte, an evaluation of the internal standard used was made. In this study, the hydrochlorothiazide and chlortalidone patterns were tested, which also ionize in a negative way, with hydrochlorothiazide being chosen as an internal standard. The second stage of the process was to optimize the chromatographic conditions. Four factors were considered important when choosing the chromatographic column: retention time, peak shape, analyte response and internal standard. With that information, the following columns were tested in the development of the method: ACE C18 column 100 mm x 4.6 mm x 5.0 µm, ACE C18 column 150 mm x 4.6 mm x 5.0 µm, ACE Excel C18 PFP column 150 mm x 4.6 mm x 5.0 µm and the ACE Phenyl column 150 mm x 4.6 mm x 5.0 µm.

Multiple mobile phases and stationary phases were examined in our study. Regarding the mobile phase, different compositions of methanol, acetonitrile in different proportions in the organic phase (B) and water with 0.1% formic acid, water with 0.2% acetic acid and water with 5 mM ammonium acetate in the aqueous phase (A) were tested. Different proportions of Phase A and B were also tested (20:80, 30:70, 35:65 (v/v), respectively).

An isocratic mobile phase, 35:65 (*v/v*) mixture of 0.2% acetic acid (A) and acetonitrile (B) was employed for the elution of efavirenz and IS and the best chromatographic profile was obtained with an ACE Phenyl 150 mm x 4.6 mm x 5 µm column. phenyl stationary phase was selected considering its ability to have π-π interactions with the electron rich double bonds of aromatic analytes, with selectivity to obtain separation from the endogenous matrix components. Other studies employed gradient elution chromatographic profile.

Regarding the liquid-liquid extraction process, extraction solvents were tested in different compositions: 100% MTBE, MTBE: Ethyl Acetate (1:1) and MTBE: Ethyl

Acetate (7:3) and the solvent with the best performance for the method was 100% MTBE.

Currently, many of the drugs under development have low water solubility (49), which can lead to difficulties in dissolution rate and, consequently, in its bioavailability. As efavirenz is a poorly water-soluble drug, belonging to class II of the biopharmaceutical classification system (50), it is a relevant case for the search for technological solutions that allow a more uniform and prominent dissolution and a bioavailability that ensures relevant plasma levels. For the investigation of different strategies that explore formulations with higher bioavailability, it is necessary to have an analytical methodology that allows the quantification of efavirenz in a biological matrix, which is sensitive and presents a short analysis time. This article presents this methodology by coupling high performance liquid chromatography with mass spectrometry. The short chromatographic run time is one of the differentials of this methodology that we have developed and validated, with a quantification time of less than four minutes, allowing the determination of several samples in a short analysis time. Curley et al. (35) and Barreiros et al. (45) described analytical run times of 8 min and 9 min, respectively, which makes this work even more attractive. The longer the analysis time, the greater the expense with solvent and the greater production of waste. The major advantage of the method developed here is the short period of analysis (< 4 min) with no impact of matrix effect.

Analytical interference can be caused by presence of some endogenous (salts, lipids, proteins, phospholipids, metabolites) or exogenous substance (co-medications, anticoagulants, excipients) and that could co-elute with the analyte of interest. This source of laboratory errors has potential to cause serious harm for the patient (51). Accumulation of lipoproteins in the patient sample can interfere with measured analytes by physical and chemical interactions (52). In addition to the normal matrix, our method investigated the matrix effect on hemolyzed and hyperlipidemic plasma samples and no interference in normal, hemolyzed or lipemic matrix was observed (4.80-8.26%).

The analytical method was developed and validated for the purpose of evaluating a new dissolution and bioavailability enhancement system based on efavirenz. Even though it is a drug with less clinical relevance in HIV-positive patients today, its use is still prescribed by the World Health Organization (WHO) itself for specific situations. Furthermore, as a class II drug according to the Biopharmaceutical Classification System, efavirenz can be seen as an excellent model for evaluating formulations aimed at solving the enormous challenge faced by the pharmaceutical industry regarding poorly water-soluble drugs. An already published study (6) was able to demonstrate a significant increase in dissolution and bioavailability of efavirenz from the microcrystals developed. Furthermore, this type of formulation meets the most up-to-date WHO recommendations regarding the development of pediatric antiretroviral formulations, focusing on oral solids, in detriment of liquid formulations, which have stability, transport and palatability problems, often resulting in lower clinical adherence to treatment by HIV-positive children.

Conclusions

A highly sensitive LC-MS/MS method was successfully developed, validated and applied to a pharmacokinetics study of efavirenz orally administered to rats. The short analysis time developed in this method is of great value because it is suitable for routine use in the laboratory. There are a lot of analytical methods for determination of efavirenz with longer time analysis and this method can be an alternative to be employed in routine pharmacokinetics analysis. In addition, this method demonstrated an ability to analyze efavirenz in both normal and hemolyzed or lipemic samples, important situations to be considered in the analysis of routinely received samples.

Conflict of interest

The authors declare no conflicts of interest.

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