



# Simple Reversed-Phase High Performance Liquid Chromatographic Estimation of the Antiretroviral Agent Efavirenz from Human Plasma

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## Authors' contributions

All authors contributed in ensuring the completion of this research work. All authors read and approved the final manuscript.

Research Article

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## ABSTRACT

**Aims:** Sequel to the resurgence of TB co-infection in HIV/AIDS patients in sub-Saharan Africa, efavirenz has become an important component of the highly active antiretroviral treatment (HAART). The objective of this study therefore is to provide a simple reversed-phase high performance liquid chromatographic (HPLC) method for the determination of efavirenz in human plasma.

**Study Design:** Method development and experimental study.

**Place and Duration of Study:** School of Pharmacy, University of Nairobi, Nairobi, Kenya, between October 2009 and September 2010.

**Methodology:** A 500 $\mu$ l drug-free plasma sample was each placed in six different centrifuge tubes (2ml) and varying aliquots of the stock solution (100 $\mu$ g/ml) of efavirenz were spiked and vortexed for 60sec to give concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 and 16 $\mu$ g/ml for calibration standards and 2.0, 4.0, 8.0 and 16.0 $\mu$ g/ml for quality control

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samples. The off-column sample pretreatment was carried out by protein precipitation using ice-cold acetonitrile. The samples were chromatographed in a phenomenex (C<sub>18</sub>) 5µm particle size column with 250x4.6mm I.D and UV detection at 254nm using a mobile phase, which was made up of a mixture of solutions A and B. Both consisted of acetonitrile, 25mM ammonium acetate buffer and glacial acetic acid in proportions of 90:10:0.1 and 10:90:0.1(v/v), respectively. The analytical technique was validated for precision, accuracy and analyte recovery.

**Results:** The calibration plot for efavirenz was found to be linear over the concentration range of 0.5 to 16.0µg/ml with the regression line equation obtained as  $y=26842x-409.4$  and the regression coefficient ( $R^2=0.999$ ), which allows for accurate reading of the concentrations of the test samples. The RSD (%) in intraday and interday assays ranged from 0.44 to 0.78%. Accuracy ranged from 92 to 110% and the recovery was >97%.

**Conclusion:** This new HPLC method is simple, reproducible and cost-effective and can be used for therapeutic drug monitoring of efavirenz in HIV/AIDS patients on HAART as demonstrated in this study.

*Keywords: Efavirenz; HPLC; pharmacokinetics; plasma; HIV/AIDS.*

## 1. INTRODUCTION

The introduction of the highly active antiretroviral therapy (HAART) regimen in sub-Saharan Africa countries has advanced the clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection. The HAART regimen consists mainly of two nucleoside analogue reverse transcriptase inhibitors and a non-nucleoside reverse transcriptase inhibitor (NNRTI) [1]. The most commonly used NNRTI is nevirapine because of its efficacy and cost-effectiveness. However, efavirenz has found increased use in HIV/AIDS patients because of the resurgence of TB co-infection as well as in patients who developed adverse reactions to nevirapine [2,3].

Efavirenz is mainly metabolized by Cytochrome P4502B6 (CYP2B6) enzyme, which also plays a significant role in the biotransformation of several therapeutically important drugs in humans, including the cytostatic prodrug, cyclophosphamide; the antidepressant, bupropion; the narcotics, ketamine, propofol and the antimalarial, artemisinin [3-5]. The plasma concentration of efavirenz is associated with a long steady state half-life of 40-55 h, which makes it suitable for once daily dose [6,7]. The development of adverse reactions is less likely with efavirenz in TB co-infection since the commonly used anti-TB drug rifampicin is a major inducer of CYP3A4 to which nevirapine is a substrate when compared to the efavirenz that is mainly metabolized by CYP2B6. Efavirenz can induce its own metabolism and this auto-induction of metabolic process was observed to give a three-fold increase in oral clearance after multiple drug administration [8]. Besides, rifampicin is reported to reduce efavirenz plasma levels by 13 to 25%, which could have therapeutic implications [9]. Therefore, monitoring of efavirenz plasma levels could be useful in HAART regimen, especially in HIV-TB co-infected patients, who are being treated with efavirenz and rifampicin concomitantly.

A number of HPLC methods have been reported for separate and simultaneous determination of antiretroviral drugs in plasma [10-12], however, there are few methods for the selective estimation of efavirenz in plasma. Some methods need special type of column for separation of analyte [13-15] while other reported techniques require a solid-phase

extraction and/or use of a gradient elution [16,17] most of which are not routinely available in sub-Saharan hospitals or laboratories setting. Therefore, this study is aimed at developing a simple, selective and cost-effective HPLC method for the quantitative estimation of efavirenz in human plasma in a resource limited setting.

This method also describes the optimization of the instrumental parameters as well as the extraction procedure from human plasma samples. The method was validated by evaluating the precision, accuracy and recovery parameters as mentioned in regulatory guidelines [18]. The proposed method was applied to evaluate the impact of CYP2B6 genetic polymorphisms on the efavirenz plasma levels in HIV/AIDS patients undergoing HAART in an African population.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

Efavirenz, (S)-6-chloro-4-(cyclopropylethynyl)-1, 4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazin-2-one (Fig. 1) standard was procured from Sigma Aldrich (St. Louis, MO, USA). Chloroform, acetone, acetonitrile, methanol, sodium hydroxide, potassium dihydrogen phosphate were products of Sigma Aldrich. Other reagents include methylated spirit (Fischer Scientific), glacial acetic acid, and ammonium acetate (BDH) (Poole, UK). All solvents were of HPLC grade while the chemicals were of analytical grade.

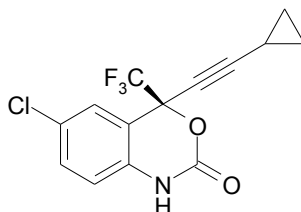


Fig. 1. Chemical structure of efavirenz

### 2.2 Chromatographic Conditions and Equipment

The HPLC system used was a Merck-Hitachi Interface D-7000 series instrument (Merck-Hitachi, Japan) made up of quaternary pumps L-7100 fitted with a gradient mixer (Merck-Hitachi), a system purge and a variable wavelength (200-800nm) ultraviolet-visible detector model L-7400 (Lachrom, Merck-Hitachi) and an 18 $\mu$ L flow cell. Injection was by a Rheodyne model 7725 valve (Cotati, California, U.S.A.) fitted with a 20 $\mu$ L loop. The system is fitted with on-line auto-sampler L-7200 and column oven L-7350 (Merck-Hitachi). The column used was a phenomenex (C<sub>18</sub>) 5 $\mu$ m particle size column and 250 x 4.6 mm I.D, reversed-phase stainless steel (Gemini) fitted to a security guard column. The mobile phase was made up of a mixture of solution A and solution B. Both solutions A and B consisted of acetonitrile, 25 mM ammonium acetate buffer and glacial acetic acid in proportions of 90:10:0.1 and 10:90:0.1 (v/v), respectively. The mobile phase was then pumped through the column at a ratio of 70:30 of solutions A and B, respectively, with a flow rate of 1ml/min and the analytical run was performed at a column oven temperature of 30°C.

### **2.3 Preparation of Stock Solutions**

The initial stock solution of 100µg/ml efavirenz was prepared by dissolving 10mg of efavirenz in water/acetonitrile (50:50, v/v). This stock solution was stored at -20°C and thawed on the day of analysis. The stock solution was serially diluted with 50% acetonitrile to give six working concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0µg/ml of the drug.

### **2.4 Sample Preparation and Extraction Procedure**

To 500µL of plasma placed in a 2 ml centrifuge tube, 1 ml of ice-cold acetonitrile was added and vortexed for 10 sec; this was followed by centrifugation at 20,000g for 10 min. Off-column sample pretreatment was carried out by protein precipitation and a 1000 µL supernatant was then transferred into separate auto sampler vials and 50 µL aliquot was injected onto the HPLC.

### **2.5 Preparation of Calibration Standards and Quality Control Samples**

A 500µl drug-free plasma sample was each placed in six different centrifuge tubes (2ml) and varying aliquots of the stock solution (100µg/ml) of efavirenz were spiked and vortexed for 60 sec to give concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 and 16µg/ml for calibration standards and 2.0, 4.0, 8.0 and 16.0 µg/ml for quality control (QC) samples. External standardization method was used to determine the standard curve and sample clean-up was carried out by protein precipitation as previously described. The peak area was plotted against the concentration of efavirenz injected. Linear regression analysis was carried out to obtain the regression equation and correlation coefficient of efavirenz using Microsoft excel version 2007.

The limit of detection taken as a concentration giving a peak as at least five times the baseline noise. The lower limit of quantitation (LLOQ) was determined as the lowest concentration of the compound which can be quantitatively determined with a precision of 20% and accuracy of 80–120% according to the FDA guidelines for bioanalytical method validation [16].

### **2.6 Analytical Precision, Recovery and Accuracy**

The assay procedure described under the preparation of calibration standards and quality control was repeated five times for QC samples within the same day (intra-day precision) and five times over four days (inter-day precision) both expressed as relative standard deviation (RSD) % values.

The recovery was computed by comparing peak areas obtained after extraction of QC samples from plasma with peak areas resulted after injecting working standard solutions at the same theoretical concentrations.

The accuracy of the measurement was assessed based on replicate analysis of four QC samples of efavirenz in each run.

## **2.7 Selectivity and Specificity**

To ensure that the proposed analytical method, which was applied to evaluate efavirenz plasma levels in HIV/AIDS patients on HAART regimen with a concomitant administration of a number of drugs, was free from interferences, the levels of some nucleoside analogues and endogenous compounds with potential for interference were evaluated. Various antiretroviral compounds such as stavudine, lamivudine, nevirapine, zidovudine, abacavir, and tenofovir were determined. Also, the selectivity of the method vis-à-vis some co-administered drugs (sulfamethoxazole, trimethoprim, sulfadoxine, pyrimethamine, rifampicin, folic acid, paracetamol) were evaluated. Two drug-free plasma samples spiked with different efavirenz concentrations followed by extraction and analysis as described in section 2.5 for each co-administered drugs was carried out.

## **2.8 Application of the Analytical Method**

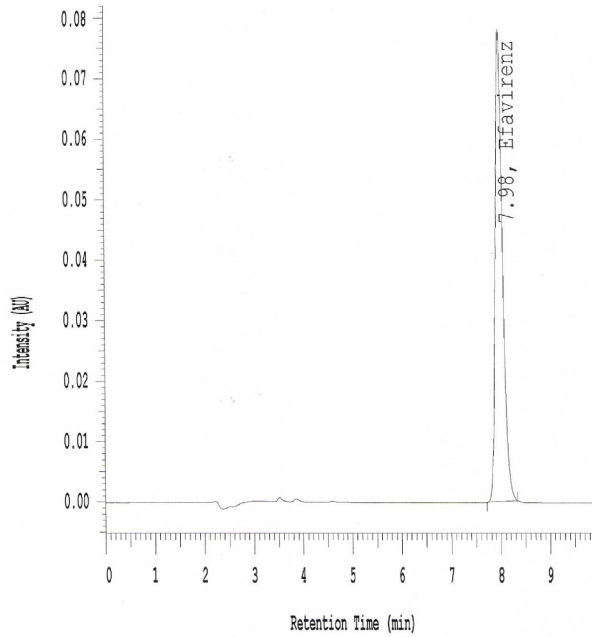
Single 5.0ml blood samples were collected by veno-puncture into EDTA, BD vacutainer tubes (Plymouth, UK) from 106 HIV/AIDS sero-positive patients receiving a once daily dose of 600 mg efavirenz in conjunction with two nucleoside reverse transcriptase inhibitors, stavudine and lamivudine, zidovudine and lamivudine and tenofovir and lamivudine at 12 to 16 h post dose administration of 600 mg efavirenz in participants who have received the drug for at least 3 weeks. Plasma was prepared from 3.0ml of whole blood by centrifugation at 10, 000g for 10 min. The plasma was stored frozen at -40°C until analyzed for the drug. The remaining 2.0 ml of whole blood was stored frozen at -40°C for genotyping procedures.

## **2.9 Statistical Analysis**

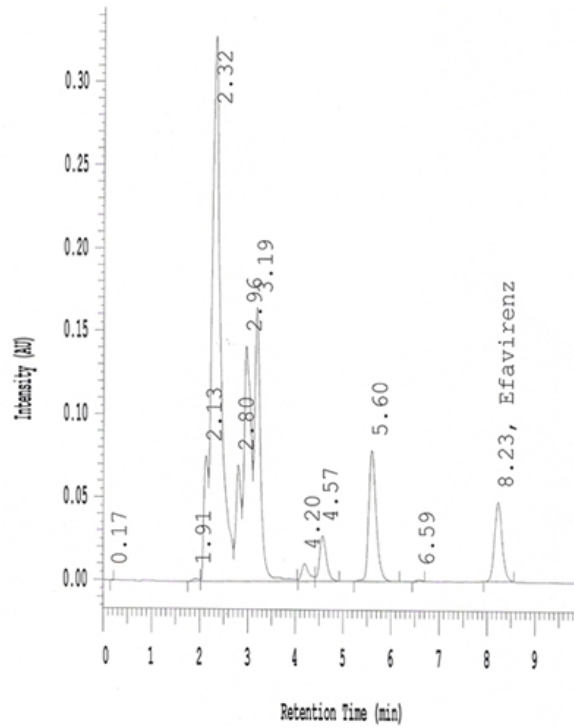
Results were expressed as mean  $\pm$  SD and RSD (%) for the precision and recovery assays as well as statistical analysis were carried out using GraphPad InStat Software version 2 and Microsoft Excel windows vista 2007 (Microsoft Corporation, USA).

## **3. RESULTS**

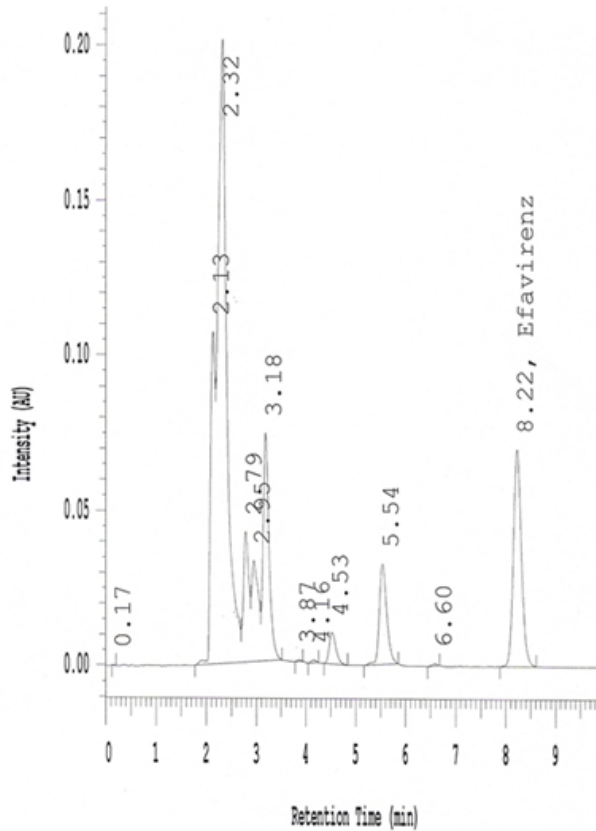
Fig. 2 shows a typical chromatogram obtained from a blank plasma sample spiked with 4 $\mu$ g/ml of efavirenz on direct injection while Figs. 3 and 4 are representative chromatograms depicting two different participants with low and high plasma levels of efavirenz, respectively. The retention time of efavirenz was approximately 8 min. These demonstrate that chromatographic peak of efavirenz was well resolved with no interferences with endogenous substances in the matrix. Also, there were no interferences from the peaks of other concomitant medications such as, stavudine, lamivudine, zidovudine and tenofovir as well as sulfadoxine, sulfamethoxazole, trimethoprim, pyrimethamine, rifampicin and folic acid. The limit of detection taken as a concentration giving a peak as at least five times the baseline noise was 20 ng/ml for efavirenz using 1ml sample, while the value of the lower limit of quantitation (LLOQ) calculated according to the FDA guidelines for bioanalytical method validation [18] was 0.2  $\mu$ g/ml.



**Fig. 2. HPLC chromatogram of spiked blank plasma with 4µg/ml of reference efavirenz**



**Fig. 3. HPLC chromatogram of test plasma of efavirenz after protein precipitation in a Subject depicting low plasma level**



**Fig. 4. HPLC chromatogram of test plasma of efavirenz after protein precipitation in a Subject depicting high plasma level**

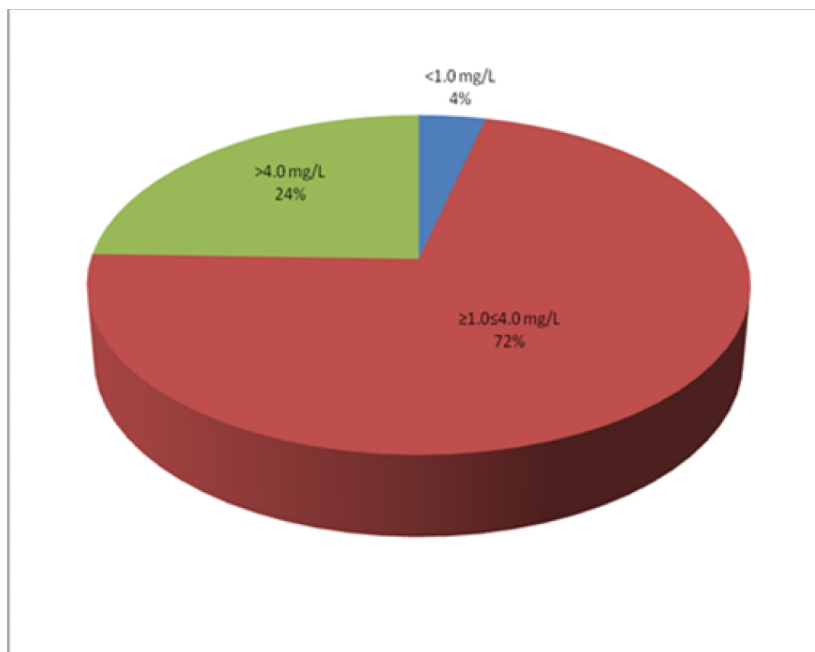
The external standardization plot for reference efavirenz in plasma was found to be linear over the concentration range of 0.5 to 16.0 µg/ml with the regression line equation obtained as  $y = 26842x - 409.4$  in line with the Beer-Lambert's law. The regression coefficient ( $R^2=0.999$ ) allows for accurate reading of the concentrations of all the test samples. The RSD%, which is a measure of the precision ranged from 0.49 to 0.78% for intra-day run and 0.44 to 0.67% for inter-day assay of efavirenz in plasma as shown in Table 1. The recovery of efavirenz from spiked plasma on protein precipitation ranged from 97.5 to 108.9%, while the accuracy of the analytical method ranged from 92 to 106% as shown in Table 1.

The steady state plasma concentrations of efavirenz were assessed from a total of 106 HIV/AIDS out-patients comprising of 40 males and 66 females. Of the 106 HIV/AIDS patients investigated, 4 (~4%) had efavirenz plasma levels below 1 mg/L, predicted to be the minimum effective concentration and 76 (~72%) had plasma concentrations above the predicted maximum safe concentrations of 4 mg/L, whereas, 26 (~24%) had plasma levels within the predicted lower and upper limit of the safety margin (i.e., >1mg/L<4mg/L) as shown in Fig. 5.

**Table 1. Intra- and inter-day assay precision, accuracy and recovery of efavirenz in plasma**

Sample	Expected conc. ( $\mu\text{g/ml}$ )	Intra-day precision ( $n = 5$ )			Inter-day precision ( $n = 5$ )			Analyte recovery ( $n = 5$ )	
		Mean Observed conc $\pm$ SD ( $\mu\text{g/ml}$ )	RSD (%)	Accuracy (%)	Mean observed conc $\pm$ SD ( $\mu\text{g/ml}$ )	RSD (%)	Accuracy (%)	(%) $\pm$ SD	RSD (%)
Efavirenz	2.0	1.92 $\pm$ 0.015	0.78	95	1.95 $\pm$ 0.01	0.51	95	97.5 $\pm$ 4.1	4.2
	4.0	4.34 $\pm$ 0.031	0.71	106	4.52 $\pm$ 0.02	0.44	103	106.5 $\pm$ 4.2	3.9
	8.0	8.05 $\pm$ 0.045	0.55	105	8.02 $\pm$ 0.054	0.67	102	102.4 $\pm$ 2.5	2.4
	16.0	16.80 $\pm$ 0.082	0.49	92	16.87 $\pm$ 0.075	0.44	92	108.9 $\pm$ 3.8	3.5





**Fig. 5. Pie chart showing the frequency of the steady state plasma concentration (mg/L) of Efavirenz in HIV/AIDS patients**

#### **4. DISCUSSION**

The analytical assay method gave a good resolution of efavirenz with no interferences from other concomitantly administered anti-retroviral drugs and endogenous compounds in all the plasma samples used in the study, thus facilitating accurate determination of the drug. The off column pretreatment of samples employed in this study produced clear supernatants from plasma free of matrix effect. Efavirenz in all the plasma samples was completely resolved to baseline and samples could be injected at 3-min intervals. External standardization was employed in this method to avoid the possibility of interference from the internal standard as the HIV/AIDS patients involved in the study were on a minimum of six different medications.

The RSDs calculated for efavirenz in the intraday and interday assays were < 1%, which indicates that the method has a high degree of precision. Extraction recovery of efavirenz from plasma was ≥ 92% for both low and high concentrations employed, which shows that the sample preparation and extraction procedure were very effective for the drug. The accuracy of the analytical method is evident in the high value range of between 92 and 106% for the drug in both low and high concentrations. These findings indicate that the analytical method developed in this study exhibits a high degree of reproducibility and accuracy.

The liquid-liquid extraction procedure and protein precipitation employed as necessary panacea for simple off-column sample clean up were key to the veracity of this analytical method when compared with some reported methods, which are time consuming and cost-ineffective. For example, the use of expensive disposable cartridges at the solid-phase drug

extraction and gradient elution control [14,15]. The mobile phase A consisted of 90% acetonitrile while mobile phase B is made up of mainly (90%) ammonium acetate buffer, which are commonly available and affordable, thus, making the method cost-effective. The rapidity of the method is underlined by the relatively short analysis time with a maximum run length of 9 min, which is much shorter than the run length time of 40 to 60 min in some reported methods [19,20]. The simplicity of this method is comparable to the method of Ramanchadran et al [21], however, the latter suffers from low sensitivity.

This method was applied to evaluate the efavirenz steady state plasma levels in HIV/AIDS out-patients. The results indicated that of the 106 HIV/AIDS patients investigated, 4 (~4%) had efavirenz plasma levels below 1 mg/L, predicted to be the minimum effective concentration and 76 (~72%) had plasma concentrations above the predicted maximum safe concentrations of 4 mg/L, whereas, 26 (~24%) had plasma levels within the predicted lower and upper limit of the safety margin (i.e. >1 mg/L<4 mg/L) [22]. Gender influence was observed in terms of plasma concentrations as no female patients had suboptimal plasma levels compared to 10% of their male counterpart. Approximately, 29% of women against 18% men had plasma concentrations within the predicted therapeutic range. Both sexes had over 70% plasma levels above the predicted upper limit of safety. This study agrees with previous findings that sex as well as plasma levels could be significant covariates in a pharmacokinetic modeling of the data to demonstrate the inter-subject variability of efavirenz exposure [23]. The findings of high plasma levels above the maximum safe concentration of 4 mg/L in the HIV/AIDS patients of Kenya origin is in concordance with previous population studies suggestive of the fact that the non-functional *CYP2B6*\*6 allelic variant is common in African populations compared to Orientals and Caucasians [24,25]. The high plasma concentrations of efavirenz found in this study irrespective of sex grouping further corroborate previous findings that both African males and females may be at risk of adverse reactions from efavirenz exposure [26].

This wide inter-subject variability of efavirenz plasma levels within African populations further provided an impetus on the desirability of utilizing therapeutic drug monitoring in dose simulation for efavirenz.

## **5. CONCLUSION**

The validated HPLC method presented is simple, reliable and cost-effective assay technique for the determination of efavirenz in human plasma. The HPLC assay method can facilitate the clinical drug monitoring of efavirenz based on its plasma concentration and dose suitability in HIV/AIDS patients on HAART as demonstrated in this study. The method is also suitable for pharmacokinetic study of efavirenz in plasma.

## **CONSENT**

Written informed consent was obtained from all the participants prior to enrolment in the study.

## **ETHICAL APPROVAL**

All authors hereby declare that all experiments have been examined and approved by the kenyatta national hospital/university of nairobi (uon) ethics and research committee (knh-erc)

with a study number (p213/7/2009) and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

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## **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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