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Short communication

Simple and rapid liquid chromatography method for determination of efavirenz in plasma

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Abstract

A simple and rapid high performance liquid chromatographic method for determination of efavirenz in human plasma was developed. The method involved extraction of sample with ethyl acetate and analysis using a reversed-phase C_{18} column (150 mm) with UV detection. The assay was linear from 0.0625 to 10.0 μg/ml. The method was specific for efavirenz estimation and the drug was stable in plasma up to one month at −20 °C. The average recovery of efavirenz from plasma was 101%. Due to its simplicity, the assay can be used for pharmacokinetic studies and therapeutic drug monitoring of efavirenz.

Keywords: Efavirenz; Plasma; HPLC

1. Introduction

Efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI), is being increasingly used since 1998 in association with other antiretroviral agents in the treatment of HIV infection [1]. Its long half-life allows once-daily dosing, and therefore presents an advantage for treatment compliance and efficacy [2–4]. Efavirenz undergoes extensive metabolism, mainly by the cytochrome P-450 isoenzyme, CYP2B6, which is known to exhibit extensive inter-individual variability. This could lead to heterogeneity in response to treatment. In addition, differences in efavirenz pharmacokinetics between various racial/ethnic groups have been reported [5–8].

Efavirenz is the preferential choice to treat HIV-tuberculosis (TB) co-infected patients. Management of both these diseases is complicated due to pharmacological drug–drug interactions between efavirenz and rifampicin, which is a first-line anti-TB drug and a potent inducer of the cytochrome P-450 system. Rifampicin is reported to reduce efavirenz plasma levels by 13 to 25%, which could have therapeutic implications [9]. The low genetic barrier of efavirenz could induce a high level of phenotypic resistance [10]. The emergence of efavirenz-resistant mutants is likely to be facilitated by repeated exposure to subtherapeutic drug levels. It has been suggested that efavirenz plasma levels can serve as predictors of treatment failure in HIV-infected patients [11–14]. Treatment failure and central nervous system side effects were found to be associated with low and high efavirenz plasma levels [15]. Therefore monitoring of efavirenz levels in plasma could be useful in the clinical management of HIV disease, especially in HIV-TB co-infected patients, who are being treated with efavirenz and rifampicin concomitantly.

A number of high performance liquid chromatography (HPLC) methods for estimation of plasma efavirenz alone [16–20] and in combination with other antiretroviral agents [21–31] have been reported. While some of these methods involve cumbersome and lengthy extraction procedures [19,20,23,25], few other methods have utilized a complex mobile phase [21,25] or gradient mobile phase [22,29]. Sample pretreatment by liquid-liquid extraction with diethyl ether [16,18,22,30,31] or protein precipitation with acetonitrile [17,27] has also been carried out in some methods. Most of these methods have used a 250 mm column, where the run time

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is expected to be higher than using a 150 mm column. The use of ethyl acetate as an organic solvent to extract efavirenz from human plasma has not been reported. While some of the published methods have not studied interference of anti-tuberculosis drugs in the assay [16,31], others have included only pyrazinamide and rifampicin in their specificity experiment [17,18,27]. It is, however, important that interference due to other first-line anti-tuberculosis drugs such as isoniazid, ethambutol and streptomycin are also ruled out. We have developed a simple method that involves extraction of efavirenz with ethyl acetate and analysis using a 150 mm column with UV detection.

The aim of this study was to develop and validate a simple and rapid HPLC assay for measuring efavirenz levels in plasma, which is free of interference from certain antiretroviral and anti-TB drugs and other co-medications that are commonly used to treat HIV/AIDS patients. After validation, the method was evaluated in samples collected from healthy volunteers who were administered a single oral dose of efavirenz.

2. Experimental

2.1. Chemicals

Efavirenz tablets were obtained from Ranbaxy (India). Pure efavirenz powder was a kind gift from Aurobindo Pharma, Hyderabad, India. Nefazodone (serzone) tablets were obtained from Bristol-Myers Squibb Company, NJ, USA. Acetonitrile (HPLC grade) was from Merck (India) and potassium dihydrogen orthophosphate was from Qualigens (India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma was obtained from Lions Blood Bank, Chennai, India.

2.2. Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), diode array detector (SPD-M10Avp) and system controller (SCL-10Avp). A rheodyne manual injector (Rheodyne, Cotati, CA, USA) attached with a 20 μL sample loop was used for loading the sample. ClassVP-LC workstation was used for data collection and acquisition. The analytical column was a C_{18}, 150 mm × 4.6 mm ID, 5 μ particle size (Lichrospher 100 RP-18e, Merck, Germany) protected by a compatible guard column.

The mobile phase consisted of 10 mM phosphate buffer pH 2.4 (adjusted with 1N HCl) and acetonitrile (55:45, v/v). Prior to preparation of the mobile phase, the phosphate buffer and acetonitrile were degassed separately using a Millipore vacuum pump. The UV detector was set at 245 nm. The chromatogram was run for 10 min at a flow rate of 2.4 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r).

2.3. Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving efavirenz in methanol. The working standards of efavirenz in concentrations ranging from 0.0625 to 10.0 μg/ml were prepared in human plasma.

2.4. Sample preparation

To 250 μl each of calibration standards and test samples, 25 μl of nefazodone (internal standard) was added at a concentration of 125 μg/ml. One milliliter of ethyl acetate was added and the contents were vortexed vigorously, centrifuged at 1000 × g and 500 μl of organic phase was evaporated to dryness. The dried residue was reconstituted in 50 μl of mobile phase and 20 μl was injected into the HPLC column.

2.5. Accuracy and linearity

The accuracy and linearity of efavirenz standards were evaluated by analysing a set of standards ranging from 0.0625 to 10.0 μg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

2.6. Precision

In order to evaluate the precision of the method, four different concentrations of efavirenz (0.25, 1.5, 3.0, 7.5 μg/ml) were prepared in pooled human plasma and analysed in duplicate on three consecutive days.

2.7. Recovery

Varying concentrations of efavirenz (0.125, 1.0, 5.0, 10.0 μg/ml) were prepared in drug-free human plasma and extracted as described above along with the internal standard. The percentage of the drug recovered from the plasma samples was determined by comparing the peak height ratio after extraction with those of unextracted methanolic solutions containing same concentrations of efavirenz as in plasma. Recovery experiments were carried out on three different occasions.

2.8. Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples obtained from six each of male and female subjects. Interference from certain antiretroviral drugs, namely, nevirapine, zidovudine, didanosine, stavudine, lamivudine, indinavir and nelfinavir, anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin and other commonly co-administered medications such as ofloxacin, acetozolamide, loperamide, prednisolone, diphenyl hydantoin, amitriptyline, cotrimoxazole and fluconazole at a high concentration of 50 μg/ml was also evaluated.

2.9. Limits of quantitation (LOQ) and detection (LOD)

These values were estimated mathematically from the standard curve equations. The LOQ was obtained by multiplying the standard deviation (S.D.) of the *Y*-axis intercepts by 10. The LOD was equal to 3.3 times the S.D. of the *Y*-axis intercepts [32].

2.10. Stability

The stability of efavirenz in human plasma when stored at −20 °C was evaluated by assaying 10 plasma samples containing efavirenz on days 1 and 30.

2.11. Samples

Three healthy volunteers were administered a single oral dose of efavirenz (600 mg) after an overnight fast. Blood samples (5 ml) were collected predosing and at 1, 2, 3, 4, 5, 6, 10, 24 and 48 h after drug administration in heparinized containers. Plasma was separated and stored at −20 °C until assay. Estimation of plasma efavirenz in all the samples was undertaken within 24 to 48 h of blood collection. Informed written consent was obtained from the volunteers before they took part in the study.

3. Results and discussion

Several HPLC methods have been described to measure efavirenz levels in plasma for pharmacokinetic studies and therapeutic drug monitoring. A survey of these papers revealed that some of these methods involved tedious extraction procedures using solid phase cartridges [19,23], while other used complex mobile phases [21,26] or gradient mobile phase [22,29]. Matthews et al. [20] have determined efavirenz in human plasma using HPLC with post-column photo chemical derivatisation

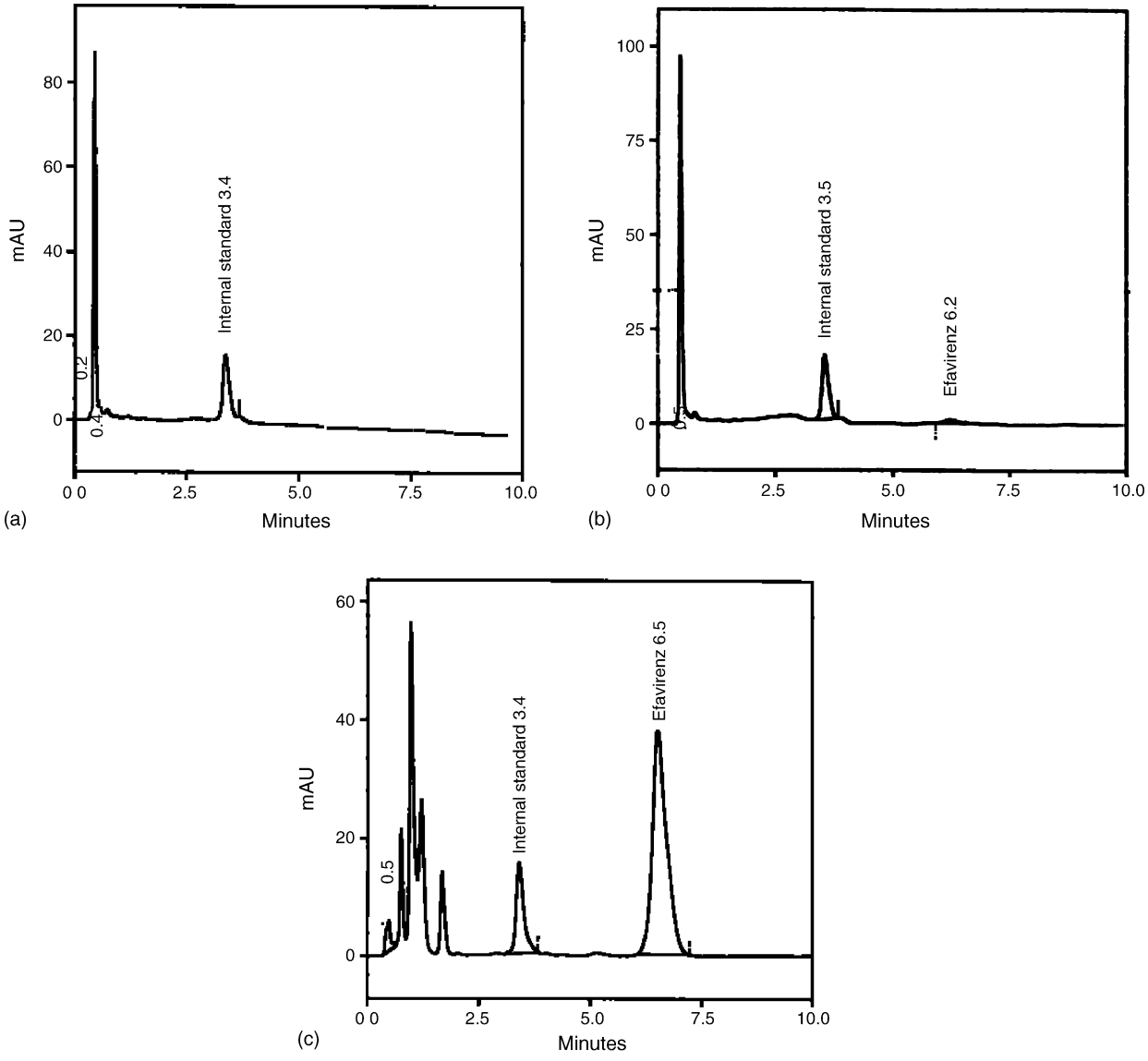

Fig. 1. (a) Chromatogram of extracted blank plasma; (b) chromatogram of extracted efavirenz plasma standard 0.0625 μg/ml; (c) chromatogram of extracted plasma sample from HIV-TB patient.

and fluorescence detection. Relatively simpler methods for sample pretreatment using liquid-liquid extraction with diethyl ether [16,18,22,30,31] or precipitation of protein with acetonitrile [17,27] have also been reported. In this study, attempts were made to extract efavirenz from plasma using ethyl acetate, and this has not been reported so far. The use of a 150 mm column resulted in reducing the run time considerably. Hence the present method has the advantages of being simple and a run time of 10 min, without any loss of analyte, and uses a small sample volume. The use of internal standard helped in monitoring the recovery of efavirenz from plasma.

Under the chromatographic conditions described above, efavirenz was well separated as seen in the representative chromatograms (Fig. 1b,c). The retention times of the internal standard and efavirenz were 3.4 and 6.2 min, respectively. Blank plasma samples did not give any peak at the retention time of efavirenz (Fig. 1a). The lowest concentration of efavirenz gave a discrete peak at 6.2 min (Fig. 1b). No endogenous substances or antiretroviral drugs such as nevirapine, zidovudine, didanosine, stavudine, lamivudine, indinavir and nelfinavir, and other commonly co-administered medications such as ofloxacin, acetozolamide, loperamide, prednisolone, diphenyl hydantoin, amitriptyline, cotrimaxazole and fluconazole interfered with the efavirenz chromatogram.

Since tuberculosis is the most common opportunistic infection in HIV-infected patients in India and other developing countries, it is essential to establish the specificity of the method with respect to anti-tuberculosis drugs, especially the first-line drugs. This is particularly important to carry out pharmacokinetic and therapeutic drug monitoring studies of efavirenz in HIV and tuberculosis co-infected patients who are receiving antiretroviral and anti-tuberculosis treatment concomitantly. None of the anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin interfered in the assay of efavirenz. This is evident from Fig. 1c, which represents the chromatogram of a plasma sample collected from a HIV-TB co-infected patient, who was being treated with efavirenz-containing antiretroviral regimens in addition to anti-TB treatment and was also receiving treatment for other opportunistic infections. The patient had developed serious adverse reactions to efavirenz, and this was confirmed by an elevated plasma efavirenz concentration of 13.12 μg/ml.

The calibration curve parameters of efavirenz from six individual experiments for standard concentrations ranging from 0.0625 to 10.0 μg/ml showed a linear relationship between peak height ratio and concentrations. The mean (±S.D.) correlation coefficient, slope and intercept values were 0.9993 ± 0.0004, 0.2703 ± 0.0108 and 0.0311 ± 0.0046, respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma efavirenz are given in Table 1. The within-day and between-day relative standard deviation (R.S.D.) for standards containing 0.0625 to 10.0 μg/ml ranged from 3.4 to 9.5% and 3.4 to 7.1%, respectively.

The reproducibility of the method was further evaluated by analysing four plasma samples containing different concentrations of efavirenz. The R.S.D. for these samples ranged from 3.3 to 6.8% (Table 2). The % variations from the actual concentrations ranged from 88 to 99%. The LOD and LOQ estimated mathematically from the standard curve equation [32] were 0.02 and 0.05 μg/ml, respectively. The absolute recovery of efavirenz from plasma extracted with ethyl acetate was calculated by comparing the peak height ratio of four concentrations of efavirenz in human plasma that underwent extraction to that of identical concentrations of efavirenz prepared in methanol without extraction. This method reliably eliminated interfering material from plasma, yielding a recovery for efavirenz that ranged from 95 to 106%.

Table 1
Linearity and reproducibility of plasma efavirenz standards

Standard concentration (μg/ml)	Mean peak height ratio ± S.D. (R.S.D.%)	
	Within day ($n = 6$)	Between day ($n = 6$)
0.0625	0.026 ± 0.002 (9.5)	0.024 ± 0.002 (7.1)
0.125	0.045 ± 0.002 (4.4)	0.050 ± 0.003 (7.0)
0.25	0.088 ± 0.006 (6.4)	0.085 ± 0.004 (4.5)
0.5	0.151 ± 0.009 (5.2)	0.142 ± 0.006 (4.5)
1.0	0.336 ± 0.011 (3.4)	0.329 ± 0.013 (3.9)
2.5	0.747 ± 0.037 (5.0)	0.739 ± 0.025 (3.4)
5.0	1.393 ± 0.082 (5.9)	1.365 ± 0.058 (4.2)
10.0	2.717 ± 0.109 (4.0)	2.491 ± 0.108 (4.3)

Table 2
Precision of plasma efavirenz assay

Actual concentration (μg/ml)	Found concentration (μg/ml) Mean ± S.D. (R.S.D.%)
0.25	0.22 ± 0.02 (6.8)
1.50	1.49 ± 0.06 (4.1)
3.00	2.79 ± 0.09 (3.3)
7.50	7.45 ± 0.24 (3.3)

The mean efavirenz concentrations measured on days 1 and 30 in 10 plasma samples were 1.39 and 1.28 μg/ml, respectively. No degradation (<10%) of efavirenz in human plasma occurred up to 1 month when stored at −20 °C.

The method described was applied for the determination of efavirenz concentrations in plasma from three healthy subjects who received a single oral dose of efavirenz (600 mg). Fig. 2 presents the mean plasma concentrations of efavirenz at various time points. Efavirenz was detectable up to 48 h after the

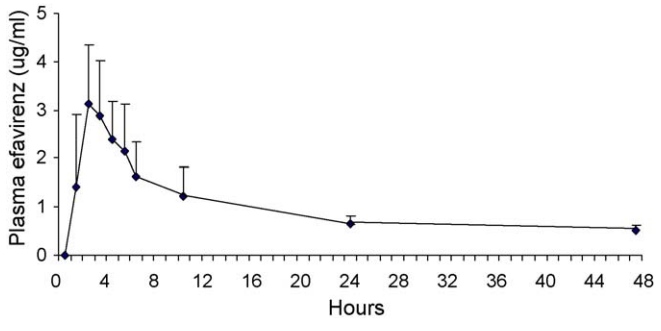

Fig. 2. Plasma efavirenz concentration in healthy volunteers. The above values are mean plasma efavirenz concentrations obtained from three healthy volunteers who were administered a single oral dose of 600 mg efavirenz. The vertical bars represent Standard Deviation.

dose. The mean plasma efavirenz concentrations at 24 and 48 h were 0.64 and 0.51 μg/ml, respectively. These values could be slightly higher at steady state. The long half life of efavirenz permits a gradual decline in its blood levels. Although the drug concentration could be detected at 48 h, it was below the therapeutic range. This is in agreement with the fact that efavirenz is prescribed once daily, so that adequate blood levels of the drug are maintained. The mean peak concentration was 3.13 μg/ml, which compared well with earlier reports [33,34]. The time to attain peak concentration was shorter in this study (2 h for all the three volunteers) than that reported by others (3–5 [33] and 3.5 h [34]). The assay spans the concentration range of clinical interest.

In conclusion, a sensitive, specific and validated method for quantitative determination of efavirenz in plasma is described. This rapid, accurate and reproducible method utilises a single step extraction. The chromatogram yields a well-resolved peak for efavirenz, with good intra and inter day precision. The easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring in patients with HIV and HIV-TB.

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