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Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

Efavirenz is a non-nucleoside reverse transcriptase inhibitor for the treatment of the HIV infection. A simple, high-performance liquid chromatographic method has been developed and validated for the quantitative determination of efavirenz in human plasma. The method involved solid-phase extraction of the drug and the internal standard (L-737,354) from 300 μl of human plasma. The analysis was via UV detection at 250 nm using a reversed-phase C_8 analytical column and a isocratic mobile phase consisting of phosphate buffer (pH 5.75)–acetonitrile that resolved the drug and internal standard from endogenous matrix components and potential coadministered drugs. Within- and between-day precisions were less than 8.6% for all quality control samples. The lower limit of quantification was 0.1 μg/ml. Recovery of efavirenz from human plasma was greater than 83%. This validated assay is being used in pharmacokinetic studies with efavirenz.

Keywords: Efavirenz

1. Introduction

Efavirenz (EFV, Fig. 1) is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) for the treatment of human immunodeficiency virus (HIV) infection. EFV has a prolonged half-life, permitting once-daily dosing. The emergence of efavirenz-resistant mutations is clearly multifactorial, but is likely to be facilitated by the repeated exposure to subtherapeutic drug levels. These variations in drug exposure may be due to drug–drug interactions, low bioavailability and inter-patient variability in drug disposition particularly variations in the activity of metabolic enzymes.

We wanted to explore the pharmacokinetic interactions between EFV and rifampin in patients with HIV-infection and tuberculosis disease. The initial approach to the problem was to develop an analytical method to determine EFV in human plasma, specifically in the presence of other drugs for the

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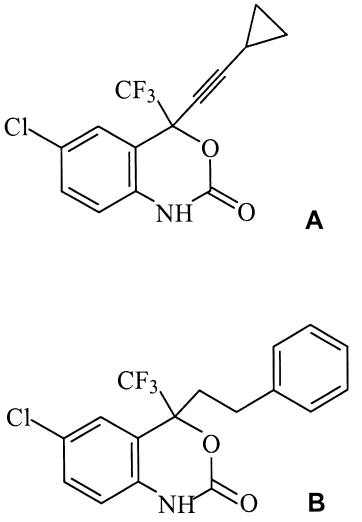

Fig. 1. Molecular structures of efavirenz (A) and L-737,345 (I.S.) (B).

treatment of tuberculosis disease or HIV. Several methodologies have been previously reported in the literature [1–5], but they use liquid–liquid extraction, and we wanted to explore the possibility of using solid-phase extraction. In this paper we describe the development and validation of a simple and sensitive high-performance liquid chromatography (HPLC) method for the quantification of EFV following solid-phase extraction of 0.3 ml of plasma.

2. Experimental

2.1. Chemicals

EFV [2H-3,1-benzoxazin-2-one,6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl), DMP 266 Sustiva] and the internal standard (I.S.) (L-737,345; Fig. 1) were kindly supplied by DuPont Pharma (Wilmington, DE, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solid-phase extraction cartridges (Oasis, 1 ml, 30 mg) were obtained from Waters (Franklin, MA, USA). HPLC-grade acetonitrile, methanol and all other reagents were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

The HPLC system consisted of the following components: a Hewlett-Packard (Palo Alto, CA, USA) Model HP1100 quaternary pump, a HP1100 degasser, a HP1100 autosampler, a HP1100 UV detector operating at 250 nm and a HP Chemstation Software Integrator.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–50 m*M* potassium phosphate with triethylamine added to 1% and adjusted to pH 5.75 with orthophosphoric acid (55:45, v/v). The elution conditions were isocratic, and the mobile phase flow-rate was set at 1 ml/min. The analytical column was a Supelcosil LC8, 3 μm particle size, 150×4.6 mm (Supelco, Bellefonte, PA, USA) with a Supelcosil LC8 (Supelco) guard column.

The sample injection volume was 80 μl. UV absorbency at a wavelength of 250 nm was used for detection.

2.4. Preparation of standards

A stock solution of EFV of 500 μg/ml was prepared in methanol. Serial dilutions of the stock solution with methanol, led to solutions of 50 and 10 μg/ml. Calibration standards in plasma covering the concentration range between 0.1 and 10 μg/ml were prepared by adding appropriate volumes of these three solutions drug-free plasma. The methanolic solutions were evaporated to dryness before adding the appropriate amount of drug-free plasma. Eight calibration concentrations were used to define the standard curve (0.1, 0.2, 0.5, 1.0, 2.5, 5, 7.5, 10 μg/ml). These calibration samples were divided into polypropylene micro-tubes as 750 μl aliquots, and frozen at −80°C until assay.

A second stock solution of EFV was used for the preparation of quality control standards in plasma (0.2, 1.0, 7.5 μg/ml). These quality controls were divided into polypropylene micro-tubes as 750 μl aliquots, and frozen at −80°C until assay.

A stock solution of I.S. (L-737,345) of 200 μg/ml was prepared in methanol and diluted to 40 μg/ml in methanol for use during sample preparation.

2.5. Sample pre-treatment

Blood samples were collected in tubes with potassium-EDTA, and centrifuged (10 min, 2000 *g*) as soon as possible. Plasma was decanted and heated for 60 min at 56°C to inactivate HIV virus, before storing at −80°C.

2.6. Sample preparation

Solid-phase extraction cartridges (Oasis, 1 ml, 30 mg) were conditioned sequentially with methanol (1 ml) and water (1 ml). An aliquot of 300 μl of the plasma sample was added to the cartridge and was allowed to pass through the bed with minimal suction. A 30-μl aliquot of the I.S. solution (L-737,345, 40 μg/ml) was added to the cartridge, followed by a water aliquot (1 ml). The columns were washed with 1 ml of 50% aqueous methanol and with 0.5 ml of 60% aqueous methanol. The bed was suctioned dry. The analytes were eluted with 0.5 ml of methanol. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature, the residue was reconstituted with 100 μl of mobile phase, and then 80 μl were injected onto the HPLC system.

2.7. Calibration and calculation procedures

Daily standard curves were constructed for EFV using the ratio of the observed peak and the I.S. heights. The unknown concentrations were computed from the linear regression equation of the peak height ratio against concentration of EFV in μg/ml. A weight factor of [1/conc.] was used.

2.8. Accuracy, precision and recovery

The accuracy and intra-day and inter-day precision of the method were estimated by assaying five replicate plasma samples at three different concentrations, in three analytical runs.

The overall mean precision was defined by the relative standard deviation (RSD) of five standards at three different concentrations analyzed on the same day. Inter-day variability was estimated from the analysis of the five standards on 3 separate days. Recovery of EFV after the solid-phase extraction was determined by comparing observed EFV peak height in extracted plasma, to those of non processed standard solutions.

2.9. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank matrices. Potential coadministered drugs (rifampin, pirazinamide, isoniazide) used in combination therapy with EFV, including HIV-reverse transcriptase inhibitors [zidovudine (AZT), lamivudine (3TC), stavudine (d4T), didanosine (ddI), zalzitabine (ddC), nevirapine, delavirdine] and protease inhibitors (indinavir, amprenavir, ritonavir, saquinavir, nelfinavir) were also analyzed and were verified to be resolved from EFV and I.S. under the HPLC conditions presented.

2.10. Stability

HIV-infected patient samples are routinely heated at 56°C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of EFV in plasma under these conditions. To verify the stability of EFV (and I.S.) in the mobile phase while waiting for HPLC analysis the extracted samples were left at room temperature for 24 h. The stability of EFV during sample handling was also verified, subjecting samples to three freeze–thaw cycles.

3. Results

3.1. Linearity

Peak height ratio of EFV and I.S. of calibration standards were proportional to the concentration of EFV in plasma over the range tested (0.1–10 μg/ml). The results of the linearity of the method are presented in Table 1. The calibration curves were

Table 1
Calibration curves

Concentration of efavirenz (μg/ml)	R1 Found (μg/ml)	R2 Found (μg/ml)	R3 Found (μg/ml)	Mean±SD	RSD (%)	Accuracy (%)
0.1	0.09	0.10	0.10	0.10±0.006	6.0	97
0.2	0.20	0.20	0.21	0.20±0.006	2.8	102
0.5	0.52	0.50	0.50	0.51±0.012	2.3	101
1.0	1.04	1.05	1.00	1.03±0.026	2.6	103
2.5	2.61	2.48	2.50	2.53±0.070	2.8	101
5.0	5.00	4.89	5.28	5.06±0.201	4.0	101
7.5	7.35	7.28	7.36	7.33±0.044	0.6	98
10	9.92	10.15	9.97	10.01±0.121	1.2	100
Slope	1.93	1.88	1.81	1.87±0.060	3.2	
y Intercept	0.036	0.018	0.014	0.023±0.012		
Correlation coefficient	0.9995	0.9997	0.9995	0.9996±0.0001		

Deviation from nominal concentration for all calibration concentrations.

fitted by linear least-squares regression and showed coefficients of determination greater than 0.999. The mean slope was 1.87 with an SD of 0.060, and an RSD of 3.2%. The mean *y*-intercept was 0.023.

3.2. Selectivity

Representative chromatograms of blank and spiked plasma samples are illustrated in Fig. 2. Blank plasma showed no interfering endogenous substances neither with EFV nor with the I.S. Potentially coadministered drugs had retention times that were different from EFV or the I.S. or were not detected with the described bioanalytical method. The retention times for the drugs tested for interference are shown in Table 2. The retention times for EFV and I.S. were 6.9 and 8.5 min, respectively.

3.3. Limit of quantification

The lower limit of quantification was defined as the concentration for which the percent deviation from the nominal concentration and the relative standard deviation were both less than 20%, as determined in the inter-day analytical runs. For EFV a concentration of 0.1 μg/ml was defined as the limit of quantification. A representative chromatogram of spiked plasma at the limit of quantification is shown in Fig. 2.

3.4. Accuracy, precision and recovery

The results from the validation of the method in human plasma are listed in Table 3. These results indicate that the method is accurate (average accuracy ranged from 102 to 109%), and precise (within-day precision ranged from 0.6 to 8.6% and between-day precision from 4.4 to 7.7%). Recovery of EFV ranged from 77 to 88%.

3.5. Stability

The stability of EFV under various conditions is shown in Table 4. Under all conditions tested, EFV is stable with detected concentrations after treatment of at least 92.1% of the initial concentration.

4. Conclusion

The analytical method described herein is currently being used for the assay of clinical study samples. Examples of plasma concentration versus time pro-

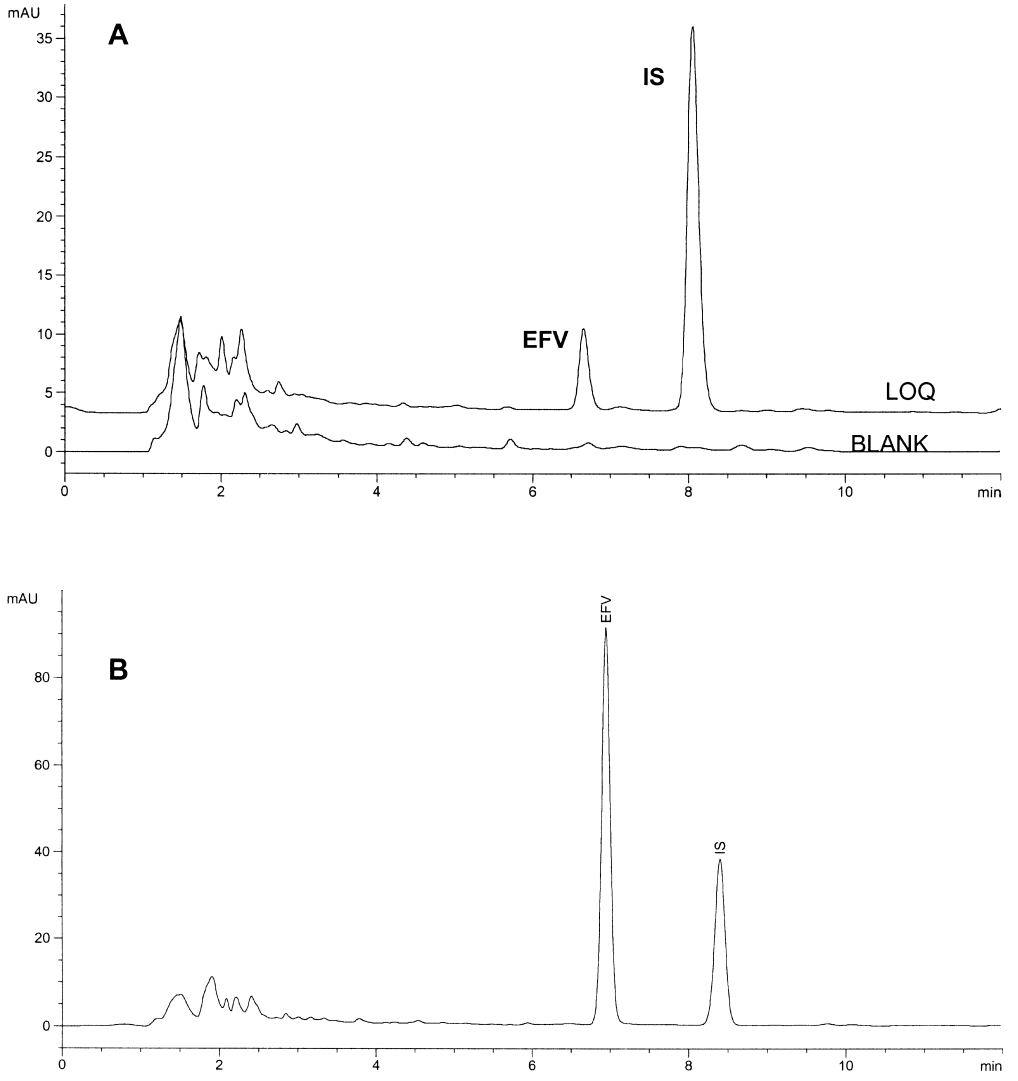

Fig. 2. Chromatograms of blank plasma and plasma spiked with 0.1 μg/ml (LOQ) of efavirenz (EFV) and internal standard (I.S.) (A) and plasma spiked with 1 μg/ml of efavirenz (EFV) and internal standard (I.S.) (B).

files in a patient receiving EFV alone (600 mg qd (once daily)) for 7 days and thereafter EFV (600 mg qd) plus rifampin (10 mg/kg/day) for 7 days more are depicted in Fig. 3. The plasmatic concentrations of EFV and rifampin are lower than those previously reported in healthy volunteers [6], probably influenced by illness. The EFV plasma concentrations when EFV is given together with rifampin are lower than when EFV is given alone as one can expect since rifampin is known to induce CYP3A4.

In conclusion, a simple, validated assay, that can readily be used in any laboratory, for the quantitative determination of EFV in human plasma is described. The assay covers the concentration range of interest and, is suitable for pharmacokinetic studies in HIV-infected patients.

Table 2
Specificity

Drug	Retention time (min)
Stavudine	ND
Didanosine	ND
Lamivudine	ND
Zidovudine	ND
Zalzitabine	ND
Nevirapine	2.2
Nelfinavir	2.8
Rifampin	3.4
Indinavir	3.2
Amprenavir	3.4
Delavirdine	3.6
Ritonavir	5.7
Pirazinamide	6.2
Efavirenz	6.9
I.S.	8.5
Saquinavir	10.9
Isoniazide	12.9

ND: Not detected.

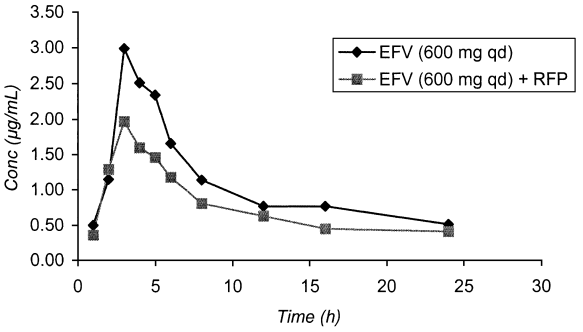

Fig. 3. Plasma efavirenz concentration–time profiles in a patient receiving 600 mg qd (once daily) of EFV alone (day 7) and in combination with rifampin (10 mg/kg/day) (day 14).

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Table 3
Accuracy and precision of the determination of EFV in plasma

	Theory (μg/ml)	*n*	Found±SD (μg/ml)	RSD (%)	Accuracy (%)	Recovery (%)
Intra-day	0.2	5	0.21±0.02	8.6	105	77.1
	1.0	5	1.02±0.08	7.0	102	88.2
	7.5	5	8.13±0.05	0.6	108	86.2
Inter-day	0.2	15	0.20±0.01	7.0	102	–
	1.0	15	1.08±0.08	7.7	109	–
	7.5	15	7.88±0.35	4.4	105	–

Table 4
Stability of EFV ($n=3$)

Storage conditions	Concentration (μg/ml)	Recovery (%)	RSD (%)
60 min at 56°C in human plasma	0.2	97.0	8.5
	1.0	92.1	3.4
	7.5	95.4	3.3
24 h at 25°C in mobile phase after solid-phase extraction	0.2	98.6	6.7
	1.0	93.7	5.2
	7.5	96.3	3.8
Three freeze–thaw cycles in human plasma	0.2	97.4	4.8
	1.0	92.9	3.3
	7.5	99.8	5.2

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