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High-performance liquid chromatographic method for the determination of HIV-1 non-nucleoside reverse transcriptase inhibitor efavirenz in plasma of patients during highly active antiretroviral therapy

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Abstract

A new high-performance liquid chromatographic method for the determination of efavirenz in human plasma is described. Quantitative recovery following liquid–liquid extraction with diethylether from 200 μl of human plasma was achieved. Subsequently, the assay was performed with 67 m*M* potassium dihydrogen phosphate–acetonitrile as a mobile phase, a XTerraRP 18 column protected with a Phenomenex C_{18} column and UV detection at 246 nm. Linear standard curves were obtained for concentrations ranging from 25 to 15 000 ng/ml. The calculated intra- and inter-day coefficients of variation were below 10%.

Keywords: Efavirenz

1. Introduction

In addition to therapy with reverse transcriptase inhibitors patients with HIV-infection are frequently treated with combinations of various HIV-1 protease inhibitors or non-nucleoside reverse transcriptase inhibitors. Efavirenz acts as a non-competitive inhibitor of the human immunodeficiency virus type 1 reverse transcriptase [1]. The high bioavailability and long plasma elimination half-life are cause of its patient friendly one-time a day dosing resulting in important drug trough levels over IC90 [1].

Efavirenz is mainly metabolized by the cytochrome P450 system, especially CYP 3A4 and CYP 2B6-isoenzymes. The drug induces its own metabolism as well as acts as a metabolic inducer of CYP3A4 and CYP2B6, which results in decreased plasma exposures of coadministered medications that are metabolized by these isoenzymes [2].

Increasing evidence for pharmacokinetic and pharmacodynamic interactions of protease inhibitors as well as patient compliance are important factors limiting the efficacy of antiretroviral therapy. In order to this therapeutic drug monitoring of protease inhibitors and of non-nucleoside reverse transcriptase

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inhibitors may be warranted to manage a patients medication regimen.

In the literature quantification methods for efavirenz use reversed-phase high-performance liquid chromatography with UV detection or mass spectrometry [3–5].

We have developed a valid, reliable and convenient HPLC-based method for the determination of efavirenz in human plasma samples, suitable for pharmacokinetic studies and routine clinical analysis in patients with HIV.

2. Experimental

2.1. Chemicals and reagents

Efavirenz (DMP 266), (*S*)-6-chloro-4-(cyclopropylethinyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one, was kindly provided by Du Pont Pharmaceuticals Company, Wilmington, Delaware, USA. The internal standard (A-86093) ((5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethylester) was kindly supplied by Abbott Inc. (Abbott Lab. North Chicago, USA).

Diethyl ether, acetonitrile, methanol, distilled water, potassium dihydrogen phosphate, sodium carbonate anhydrous, sodium hydroxide and sodium bicarbonate were products of Merck (Darmstadt, Germany). Blank, drug free plasma was obtained from Sigma–Aldrich Chemie (Deisenhofen, Germany).

All chemicals were of analytical reagent grade and all solvents were of HPLC grade.

2.2. Chromatographic equipment and conditions

The high-performance liquid chromatography (HPLC) system consisted of a Beckman System Gold (Beckman Instruments Munich, Germany) equipped with a 126 solvent delivery module, a 507 autoinjector, a 168 UV–Vis photodiode-array detector and a Beckman System Gold software for peak identification and integration.

The analytical column was a XTerra™ RP18 column (2.1×150 mm I.D., particle size 5 μm; Waters Corporation, Milford, MA, USA) protected with a security guard C 18 (4×2.0 mm I.D.; Phenomenex Inc., Hösbach, Germany). The signal was monitored at 246 nm. Peak purity tests were carried out continuously during the batch analysis. A peak controlled spectrum recording was selected with a range of 190–300 nm and a peak purity method represents the storage of one spectral scan at peak upslope, apex and downslope.

The mobile phase consisted of acetonitrile–67 m*M* potassium dihydrogen phosphate (adjusted to pH 7.4 with sodium hydroxide, 32%) at a ratio of (50:50; v/v). The flow-rate was set at 0.2 ml/min.

2.3. Standard preparation

The initial stock solution of efavirenz was prepared at a concentration of 10 mg efavirenz in 100 ml methanol. Four working solutions at concentrations of 25–15 000 ng/ml where appropriately diluted.

A stock solution of the internal standard (A-86093) was prepared in a mixture of 67 m*M* potassium dihydrogen phosphate–methanol (50:50, v/v) to yield a final concentration of 10 mg/100 ml. Each solution was stored at 4°C and stable for at least 3 months.

For preparation of the plasma standard samples a appropriate amount of the stock solutions and the internal standard (10 μg/ml) were added to blank plasma to achieve the above-mentioned calibration concentrations.

2.4. Sample preparation

A 200 μl aliquot of plasma was mixed with 400 μl carbonate buffer (0.1 *M* sodium carbonate–sodium bicarbonate, pH 9.4), furthermore an aliquot of 20 μl solution of internal standard (A86093) was added to a glass tube. After vortexing for 10 s the tubes were capped and extracted twice with 3 ml diethyl ether for 30 s, followed by centrifugation at 3000 *g* (4°C). The organic layers were transferred to a glass centrifuge tube and evaporated to dryness with a gentle stream of nitrogen at 37°C.

The residue was reconstituted in 300 μl 0.67 m*M* potassium dihydrogen phosphate–methanol (50:50,

v/v). The aqueous layer was transferred to autosampler vials with glass micro inserts for RPLC analysis. A 100 μl aliquot was injected into the HPLC system.

2.5. Specificity and selectivity

In order to evaluate levels of endogenous compounds with potential for interference with the analytical method, analysis of six different blank samples was performed. The following compounds were determined: abacavir, adefovir, amprenavir, didanosine, fluconazole, folinic acid, ganciclovir, indinavir, itraconazole, lamivudine, lopinavir, methadone, methotrexate, nelfinavir, M8-metabolite of nelfmavir, nevirapine, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, zalcitabine, and zidovudine. Three plasma samples spiked with different efavirenz concentrations were analysed for each substrate.

2.6. Limit of quantitation

The limit of detection in plasma (LOD) was defined by the lowest detectable concentration yielding a signal-to-noise ratio of three, indicating a significant difference of spiked and blank samples in plasma samples of three individuals as determined by the two-tailed, paired Student's *t*-test.

For the concentration to be accepted as the lower limit of quantitation (LOQ) the intra- and inter-batch measure of accuracy (percent deviation from the nominal concentration) and precision (relative standard deviation) are to be less than 20%. All samples were assayed in triplicates.

The upper limit of quantitation (ULQ) was arbitrarily set at 15 000 ng/ml.

2.7. Accuracy, precision, linearity and recovery

Intra-day accuracy and precision of the method were determined by measuring eight replicate plasma samples at four different concentrations of efavirenz (200, 2300, 6000 and 8500 ng/ml).

Accuracy was calculated as the relative error of the nominal concentration. Precision was expressed in terms of relative standard deviation and obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable. To obtain the inter-day accuracy and precision, four samples of each concentration were analyzed at nine different days as described above.

Daily standard curves were evaluated by duplicate analysis of eight spiked plasma samples for efavirenz in the range of 25 to 15 000 ng/ml.

A linear weighted [1/concentration squared] least squares regression analysis to plot the observed peak area/internal standard ratio of efavirenz was performed. Linearity and assay reproducibility were determined by measuring the standard concentration in five separate assay runs on five separate days.

The linearity of five calibration curves was tested with the *F*-test for lack of fit, using a weight factor of $[1/\text{conc.}^2]$.

The recovery of efavirenz and the internal standard in the extraction procedure was determined comparing the detected concentrations of efavirenz (100, 1000 and 10 000 ng/ml) in three extracted spiked plasma samples to those of non-processed standard solutions.

2.8. Analysis of patient samples

Plasma samples derived from 45 HIV-infected patients during antiretroviral therapy with combination of different NRTI zidovudine, lamivudine, stavudine or didanosine and/or protease inhibitor and efavirenz were taken 12 h after the ingestion of 600 mg efavirenz. Plasma samples of all patients were obtained by a standardized procedure. Plasma was separated by centrifugation at 3000 *g* for 10 min at 4°C and was immediately stored at −20°C until further analysis.

2.9. Calculation and data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 7.5.2.dt. (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Chromatography and detection

Sample preparation by protein precipitation with acetonitrile using a volume of 200 μl plasma was described previously [6]. We used a 200 μl aliquot of plasma following liquid–liquid extraction with diethyl ether. So an equal baseline and good sensitivity of our assay was reached. Liquid–liquid extraction further resulted in less residue in the column after a run with the result of high possible number of runs in one column. Compared to solid-phase extraction, the liquid–liquid extraction procedure seems to be more cost-effective. The use of an internal standard (I.S.) makes our assay more reliable than the previously described methods [3]. The use of A-86093 as an internal standard with a retention time of 8.60 min was found to be convenient.

Peak shape, separation from endogenous compounds and separation from other antiretrovirals, especially PI, were therefore optimized by using a XTerra™ RP 18 column (2.1×150 mm I.D., particle size 5 μm; Waters Corporation, Milford, MA, USA) protected with a security guard C_{18} (4×2.0 mm I.D.; Phenomenex Inc., Hösbach, Germany). Under these conditions, a gradient of the mobile phase as described in former methods of determination of protease inhibitors in plasma was not necessary [6,7].

A chromatogram of a blank plasma sample (Fig. 1) showed no interfering endogenous peaks. A representative HPLC run of a spiked plasma sample with 25 ng/ml efavirenz is shown in Fig. 2.

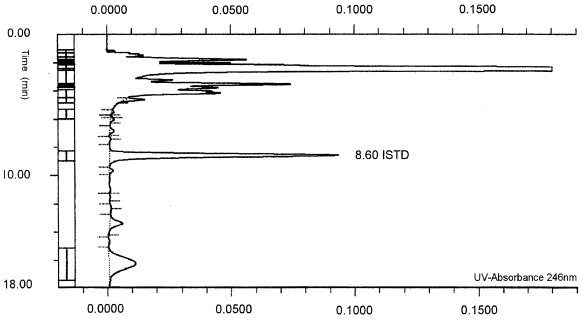

Fig. 1. Chromatogram of a blank human plasma sample spiked with 10 μg/ml internal standard A-86093 (8.60 min) with UV-detection at 246 nm.

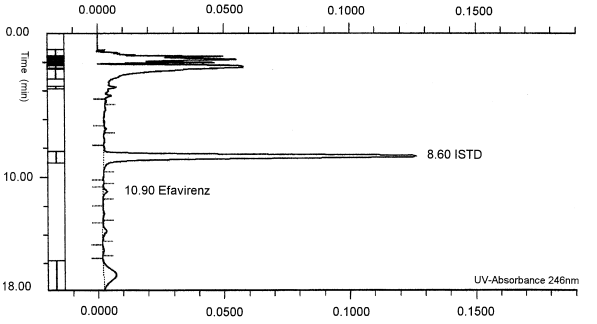

Fig. 2. Chromatogram of a blank human plasma sample spiked with 10 μg/ml internal standard A-86093 (8.60 min) and efavirenz 25 ng/ml (10.90 min) with UV-detection at 246 nm.

A HPLC run of a plasma sample derived from a patient receiving a salvage therapeutic regimen consisting of didanosine (0.4 g/day), stavudine (0.08 g/day), efavirenz (0.6 g/day) and a combination PI-therapy with saquinavir (2×0.6 g/day) and nelfinavir (2×0.75 g/day) is shown in Fig. 3. The retention time of efavirenz was 10.90 min. A plasma concentration of 2474 ng/ml was measured.

3.2. Specificity and selectivity

Drug-free plasma samples obtained from healthy individuals were devoid of interference near the retention time of efavirenz and the internal standard.

The analysis of plasma samples containing abacavir, adefovir, amprenavir, didanosine, folinic

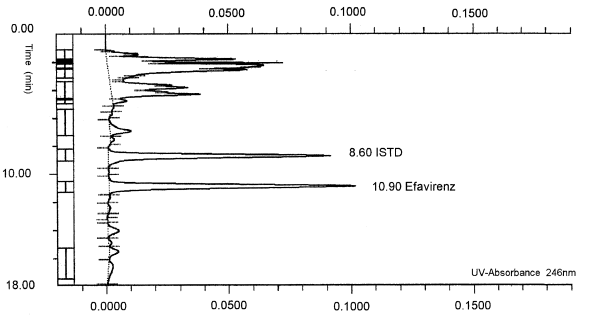

Fig. 3. Chromatogram of a patient plasma sample receiving didanosine (0.4 g/day), stavudine (0.08 g/day), efavirenz (0.6 g/day) and a combination PI-therapy with saquinavir (2×0.6 g/day) and nelfinavir (2×0.75 g/day). The retention time of efavirenz was 10.90 min. An efavirenz concentration of 2474 ng/ml was detected.

acid, fluconazole, ganciclovir, indinavir, itraconazole, lamivudine, lopinavir, methadone, methotrexate, nelfinavir, M8-metabolite of nelfinavir, nevirapine, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, zalcitabine, or zidovudine showed no interference neither with the extraction procedure nor with the analytical method.

3.3. Limit of quantitation

The detection limit of efavirenz in plasma was determined at 3 ng/ml. The lower limit of quantitation was reached at a concentration of 25 ng. The upper limit of quantitation was arbitrarily set at 15 000 ng/ml.

3.4. Accuracy, precision, linearity and recovery of the assay

The intra-day accuracy and precision of the method was determined in eight analytical runs including four different concentrations. Precision ranged from 2.0 to 3.8%. Intra-day accuracy was −1.3 to 5.3%. The results of intra-day validation are presented in Table 1. The inter-day accuracy and precision of efavirenz as shown in Table 2 were below 10%. Using the ratios of the observed peak areas for efavirenz and the internal standard in eight spiked plasma samples analyzed in duplicate, the standard curves showed a correlation coefficient of 0.997 (range 25 to 15 000 ng/ml) as determined by least-square analysis. All calibration curves proved to be linear in the respective ranges listed above in the *F*-test for lack of fit, which was performed to assess the linearity of the regression model. The C.V. % of five slopes was 4%.

Table 1
Accuracy and precision for the analysis of efavirenz (EFV) in spiked plasma samples: Intra-day precision (C.V. %) and accuracy (RE %)

Sample concentration (ng/ml)	200	2300	6000	8500
Concentration found (ng/ml)				
mean	202	2275	5969	8049
±5.0	5.3	71.8	117.4	306.4
C.V. %	2.6	3.2	2.0	3.8
RE %	−1.3	1.1	0.5	5.3
n=	8	8	8	

Table 2
Accuracy and precision for the analysis of efavirenz (EFV) in spiked plasma samples: Inter-day precision (C.V. %) and accuracy (RE %)

Sample concentration (ng/ml)	200	2300	6000	8500
Concentration found (ng/ml)				
mean	200	2242	5920	7852
±SD	6.9	102.0	278.1	400.4
C.V. %	3.4	4.6	4.7	5.1
RE %	0.06	2.5	1.3	7.6
n=	36	36	36	36

The recovery of efavirenz was estimated by comparison of peak areas in extracted spiked drug-free plasma with those of standard solutions. Recovery was found to be 96.2±3.0% (*n*=3).

No degradation occurred for 6 months when kept at −20°C or at room temperature at 48 h in reconstituted extracts as conducted in other investigations [3,4,8].

3.5. Analysis of patient samples

Plasma samples derived from 45 HIV-infected patients during antiretroviral therapy with a combination of different NRTI zidovudine, lamivudine, stavudine or didanosine and/or protease inhibitor and efavirenz were taken 12 h after the ingestion of 600 mg efavirenz. Plasma concentrations of efavirenz were 3689±2824 ng/ml. The presented results, shown in Fig. 4, demonstrate the applicabili-

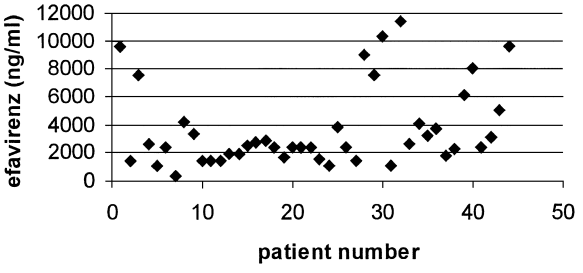

Fig. 4. Efavirenz plasma concentrations from 45 HIV-infected patients during antiretroviral therapy. Plasma samples were taken 12 h after the ingestion of 600 mg efavirenz.

ty of the assay for therapeutic drug monitoring in HIV-infected patients.

4. Conclusion

We here present a validated, reliable and convenient assay for the determination of efavirenz in human plasma. The described HPLC assay can readily be used in a standard hospital laboratory.

In our hands, the described procedure was most suitable. Calibration curves for efavirenz from 25 to 15 000 ng/ml are appropriate for clinical drug monitoring and are especially suitable for assessment of patient's adherence.

The practicability of the assay is demonstrated by plasma levels of 45 patients regularly seen in our outpatient care unit.

This HPLC method is preferentially used for drug monitoring in patients treated with efavirenz in combination with other antiretroviral agents. This type of drug monitoring may be essential for the estimation of drug plasma level in patients with loss of antiretroviral efficacy caused by interaction with other hepatically metabolized co-medications, non-compliance or resorption deficit of efavirenz in contrast to viral resistance.

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