ELSEVIER

Journal of Chromatography B, 734 (1999) 55–61

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantitative determination of efavirenz (DMP 266), a novel non-nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection

Agnes I. Veldkamp[a,*], Rolf P.G. van Heeswijk[a], Pieter L. Meenhorst[b], Jan W. Mulder[b], Joep M.A. Lange[c], Jos H. Beijnen[a], Richard M.W. Hoetelmans[a]

[a]*Department of Pharmacy and Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[b]*Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands*
[c]*National AIDS Therapy Evaluation Centre, Department of Internal Medicine, Academic Medical Centre, Amsterdam, The Netherlands*

Received 5 May 1999; received in revised form 13 July 1999; accepted 13 July 1999

Abstract

Efavirenz is a novel non-nucleoside reverse transcriptase inhibitor for the treatment of HIV-1-infected individuals. A simple and rapid high-performance liquid chromatographic method for the quantification of efavirenz in human plasma suitable for therapeutic drug monitoring in plasma is described. Sample pretreatment consists of protein precipitation with acetonitrile and subsequent evaporation of the extract to concentrate the analyte. The drug is separated from endogenous compounds by isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection at 246 nm. The method has been validated over the range of 10 to 10 000 ng/ml using a volume of 250 μl of plasma. The assay is linear over this concentration range as indicated by the *F*-test for lack of fit. Within- and between-day precisions are less than 4.3% for all quality control samples. The lower limit of quantitation is 10 ng/ml and the recovery of efavirenz from human plasma is 106.4% (±1.8%). Frequently co-administered drugs did not interfere with the described methodology. Efavirenz is stable under various relevant storage conditions, for example when stored for 24 h at room temperature. This validated assay is suited for use in pharmacokinetic studies with efavirenz and can readily be implemented in the setting of a hospital laboratory for the monitoring of efavirenz concentrations.

Keywords: Efavirenz; DMP 266

1. Introduction

Efavirenz (DMP 266, Fig. 1) belongs to the class of the non-nucleoside reverse transcriptase inhibitors. Efavirenz exerts its action by non-competitive inhibition of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. HIV-2 reverse transcriptase and human cellular DNA polymerases are not inhibited by efavirenz [1].

Treatment of patients with efavirenz monotherapy

*Corresponding author. Tel.: +31-20-5124-481; fax: +31-20-5124-753.
E-mail address: apabg@slz.nl (A.I. Veldkamp)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00336-9

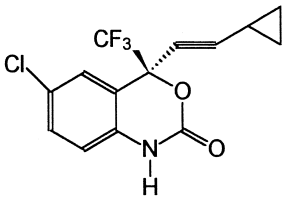

Fig. 1. Molecular structure of efavirenz.

rapidly selects for resistant isolates of HIV-1 with the mutation at reverse transcriptase amino acid position 103 (lysine to asparagine) being most frequently observed [1,2]. However, when administered in combination with nucleoside reverse transcriptase inhibitors and protease inhibitors, efavirenz has demonstrated a potent antiviral effect and is therefore an important addition to the currently available armamentarium of antiretroviral drugs [3–6].

Efavirenz is metabolized by the cytochrome (CYP) P450 isoenzymes 3A4 and 2B6 and induces metabolizing enzymes, resulting in induction of its own metabolism (autoinduction) [1,2].

Meals of normal composition have no appreciable effect on the bioavailability, compared to ingesting the drug on an empty stomach. However, it is not recommended to ingest efavirenz shortly after a high-fat meal, as the bioavailability increases with 50%, and this may increase the severity or frequency of adverse events. The currently recommended dosage for efavirenz is 600 mg once daily.

Since many drugs are metabolized by CYP 3A4 and 2B6, drug–drug interactions are likely to occur. For example, the area under the plasma concentration–time curve (AUC) of efavirenz is increased by 21% when ritonavir is coadministered, and the AUC of efavirenz decreases by 12% when saquinavir is coadministered [1,2,7].

We here report the development and validation of a high-performance liquid chromatographic assay with ultraviolet detection for the quantitative determination of efavirenz in human plasma. To our knowledge, no detailed methodology for the quantitative determination of efavirenz in plasma has been described thus far. The presented assay is useful for pharmacokinetic research and therapeutic drug monitoring in HIV-1-infected individuals treated with efavirenz.

2. Experimental

2.1. Equipment

The high-performance liquid chromatography (HPLC) system consisted of a P100 solvent delivery pump (Thermo Separation Products, Fremont, CA, USA), an AS300 automatic sample injection device (Thermo Separation Products), an UV1000 wavelength detector (Thermo Separation Products) and a Datajet integrator (Thermo Separation Products). The analytical column was a Zorbax SB C_{18} column (150×4.6 mm I.D., particle size 3.5 μm; Rockland Technologies, Newport, DE, USA) protected by a Chromguard C_{18} guard column (10×3 mm I.D.; Chrompack International, Middelburg, The Netherlands). Analytical runs were processed by Winner on Windows (version 2.0, Thermo Separation Products).

2.2. Chemicals

Efavirenz was kindly provided by The Du Pont Merck Pharmaceutical Company (Lot. 154598-52-4, Wilmington, DE, USA). Acetonitrile and methanol (both HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Potassium dihydrogenphosphate was purchased from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands).

2.3. Preparation of standards

Stock solutions of efavirenz were prepared by dissolving the appropriate amount of the compound, accurately weighed, in methanol to yield a concentration of approximately 1 mg/ml. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital, Amsterdam, The Netherlands), either as solutions for injection or after dissolving solid reference material in 50% (v/v) methanol (final concentration 500 μg/ml).

2.4. Sample pretreatment

For the preparation of calibration samples the stock solution of efavirenz was diluted with metha-

nol. For the construction of each calibration curve 11 spiked plasma samples were analyzed in duplicate. Calibration concentrations of 10–10 000 ng/ml efavirenz in plasma were prepared in Eppendorf tubes by adding 50 μl of diluted stock solution to 950 μl of blank human plasma. The solutions were thoroughly mixed for 10 s. Subsequently, 250 μl of the plasma standards were mixed with 1000 μl of acetonitrile for 30 s. The tubes were then centrifuged for 10 min at 10 500 *g*, and 1000 μl of the clear supernatant was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were then dissolved in 100 μl of mobile phase, mixed for 1 min and centrifuged for 10 min at 10 500 *g*. The clear supernatants were transferred to autosampler vials with inserts.

A second stock solution, with separate weighing of the analyte, was used for the preparation of quality control samples (QCs) yielding concentrations of 251, 1002, 4008 and 8015 ng/ml of efavirenz in plasma. The QCs were processed in an identical manner to the calibration samples.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature on the previously described C_{18} analytical column with a mobile phase composed of phosphate buffer (25 m*M*)–acetonitrile (53:47, v/v). The pH of the water–acetonitrile mixture was adjusted to 7.5 using a diluted 2 *M* solution of potassium hydroxide. Prior to use, air was removed by purging helium through the mobile phase. Absorbance was measured at 246 nm. The flow-rate was maintained at 1.5 ml/min. Aliquots of 50 μl were injected.

2.6. Specificity and selectivity

The interference of endogenous compounds was investigated by the analysis of different blank plasma samples originating from six individuals. The following compounds, frequently used by HIV-infected individuals, were investigated for interference with the analytical method (including sample pretreatment): abacavir, adefovir, amprenavir, co-trimoxazole, delavirdine, didanosine, fluconazole, folinic acid, ganciclovir, indinavir, itraconazole, lamivudine, methadone, nelfinavir, nevirapine, oxazepam, pyrazinamide, ranitidine, rifampin, ritonavir, saquinavir, stavudine, zalcitabine, zidovudine and zidovudine-glucuronide in a final concentration of 20 μg/ml in plasma.

2.7. Limit of quantitation

The lower limit of quantitation (LLQ) was defined as the concentration for which the relative standard deviation (RSD) and the deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) was arbitrarily set at 10 000 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between- and within-day precisions of the method were determined by assaying six replicates of each of the QCs in three separate analytical runs. The accuracy was calculated at each test concentration. The measured concentration was divided by the nominal concentration and multiplied by 100%. The within- and between-day precision were obtained by analysis of variance (ANOVA) for each test concentration, using the analytical run as the grouping variable.

Linearity of three calibration curves was tested with the *F*-test for lack of fit, using a weighing factor of $1/\text{conc.}^2$ [8,9]. For the construction of each calibration curve 11 spiked plasma samples were analyzed in duplicate.

The average recovery of efavirenz over the concentration range of the standard curve was determined in three analytical runs by calculating the ratio of the slopes of a calibration curve in plasma and of non-processed solutions.

2.9. Stability

Blank plasma samples were spiked with aliquots of diluted efavirenz stock solution to yield concentrations of 251, 1002, 4008 and 8015 ng/ml. These samples were kept for 1 h at 60°C, 24 h at 25°C, 7 days at 4°C, 30 days at −30°C, and 30 days at −30°C including three freeze–thaw cycles. For each concentration and each storage condition six replicates were analyzed in an analytical run. The

concentration of efavirenz after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

2.10. Analysis of patient samples

Plasma samples of 14 different HIV-1-infected individuals treated with efavirenz (600 mg once daily) were analyzed with the currently reported methodology.

2.11. Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated P values were 0.05 or less.

3. Results and discussion

3.1. Chromatography and detection

The starting-point for the development was the HPLC–UV method for the non-nucleoside reverse transcriptase inhibitor nevirapine developed in our laboratory [10]. Using this methodology, efavirenz eluted at approximately 45 min. We increased the amount of modifier in the mobile phase to decrease the retention time of efavirenz. Optimal chromatography was obtained using a Zorbax C_{18} column of 75 mm×4.6 mm I.D., particle size 3.5 μm. Unfortunately, when interference analysis was performed, ritonavir and efavirenz eluted concurrently. When we replaced the column with a Zorbax C_{18} column of 150 mm×4.6 mm I.D., particle size 3.5 μm this problem was solved and efavirenz (retention time 10.2 min) and ritonavir (retention time 8.7 min) were separated. An acetonitrile content of 47% and a pH value of 7.5 of the mobile phase turned out to be optimal for separation of efavirenz from endogenous plasma compounds. The flow-rate was set at 1.5 ml/min to decrease the runtime.

Typical chromatograms of a blank plasma sample and of a spiked sample containing 25 ng/ml efavirenz in plasma are shown in Figs. 2 and 3,

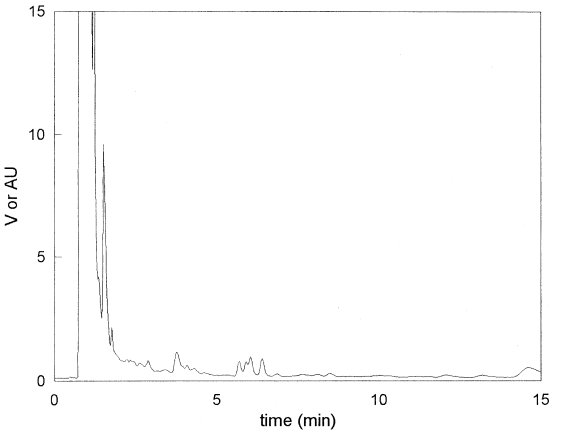

Fig. 2. Chromatogram typical for the analysis of efavirenz: blank plasma.

respectively. The run time of the HPLC assay is 15 min. The UV spectra of efavirenz in methanol and in mobile phase were recorded and maximal absorbance was measured at 246 nm which was used as the wavelength of detection.

No suitable internal standard was available. However, the assay provided satisfactory validation results without using an internal standard.

3.2. Sample pretreatment and recovery

Protein precipitation with acetonitrile and subsequent evaporation to concentrate the analyte, is a rapid, simple and effective sample pretreatment

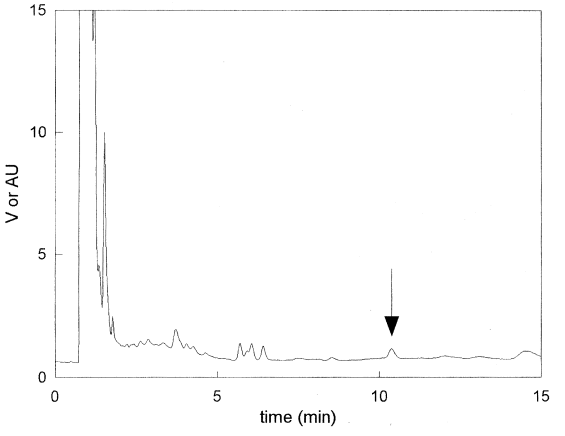

Fig. 3. Chromatogram typical for the analysis of efavirenz: a spiked plasma sample of 25 ng/ml.

procedure. Recovery of efavirenz from spiked plasma samples using this methodology is 106.4%.

3.3. Specificity and selectivity

Blank plasma from six different individuals showed no interfering endogenous substances eluting at the retention time (t_R) of efavirenz. Potentially co-administered drugs or metabolites tested had retention times that were different from efavirenz (t_R=10.2 min) (amprenavir, t_R=3.7 min, indinavir, t_R=2.8 min, itraconazole, t_R=31.4 min, methadone, t_R=14.5 min, nelfinavir, t_R=23.4 min, ritonavir t_R=8.7 min, saquinavir, t_R=12.0 min) or were not detected with the described analytical method.

3.4. Limit of quantification

The RSD and deviation from the nominal concentration were both less than 15% at a concentration of 10 ng/ml in plasma. At all other concentrations up to the ULQ (10 000 ng/ml) the RSD and deviation from the nominal concentration were always less than 13%. We defined 10 ng/ml as the LLQ.

In clinical practice, the average steady-state peak and trough concentrations are approximately 4 and 1.8 μg/ml, respectively [1,2]. Hence, the concentration range which is required for the application of this HPLC methodology in clinical pharmacokinetic studies and therapeutic drug monitoring of efavirenz is covered.

Table 2
Accuracy and precision for the analysis of efavirenz in spiked human plasma samples

Concentration (ng/ml)	Accuracy (%)	Precision		n[a]
		Between-day	Within-day	
251	95.6	1.1	3.1	17
1002	100.0	2.0	2.9	18
4008	101.7	4.3	4.3	18
8015	100.8	3.1	2.5	17

[a] n=Total number of replicates in three analytical runs.

3.5. Validation: accuracy, precision, linearity and stability

Calibration curves proved to be linear in the range of 10 to 10 000 ng/ml with the use of the F-test for lack of fit (α=0.05) as an indicator of linearity of the regression model. For every calibration curve the calibration concentrations were back-calculated from the peak areas of efavirenz (Table 1).

Accuracies and precisions for the quantitation of efavirenz in human plasma are listed in Table 2. The use of peak areas in combination with a weighing factor ($1/\text{conc.}^2$) resulted in a minimal deviation from nominal concentrations.

The method proved to be accurate (average accuracy at four concentrations 95.6–101.7% of the nominal concentrations) and precise (within-day

Table 1
Calibration curves: deviation from the nominal concentration (Dev.) and relative standard deviation (RSD) for the analyte at all tested calibration concentrations

Concentration of efavirenz	Run 1 (n=2)[a]		Run 2 (n=2)[a]		Run 3 (n=2)[a]		Mean	
(ng/ml)	Dev. (%)	RSD (%)	Dev. (%)	RSD (%)	Dev. (%)	RSD (%)	Dev. (%)	RSD (%)
10	+1.3	11.5	+2.9	4.7	+1.9	2.6	+2.0	5.7
25	−2.4	10.3	−5.6	4.3	−9.7	3.3	−6.0	6.4
50	−2.0	4.9	−5.2	1.0	+2.5	4.2	−1.6	4.6
100	−0.9	0.5	+1.4	1.7	+13.0	3.2	+4.5	6.6
250	−0.7	0.9	+2.9	3.4	+5.8	0.2	+2.7	3.2
500	+2.2	0.8	+3.5	0.5	1.2	1.7	+2.3	1.3
1000	+6.2	0.3	+2.3	2.2	−10.8	4.8	−0.8	8.3
2500	+2.9	4.0	+3.3	1.2	0.0	1.3	+2.1	2.5
5000	−1.6	1.7	−2.9	1.2	−2.4	0.3	−2.3	1.1
7500	−2.9	3.9	+0.5	0.1	+1.5	4.4	−0.3	3.3
10 000	−2.3	3.2	−3.2	1.9	−3.1	1.2	−2.9	1.8

[a] n=Number of replicates.

precision ranged from 2.5 to 4.3% and between-day precision ranged from 1.1 to 4.3%). Correlation coefficients (r^2) of calibration curves were >0.998 as determined by least-squares linear regression analysis.

Under all conditions tested efavirenz is stable with concentrations of at least 93.4% of the initial concentrations (Table 3).

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV-1-infected individuals was demonstrated by measuring 14 patient samples. In this population we found plasma concentrations between 0.14 and 7.32 μg/ml. A typical chromatogram of a patient sample of 7.0 μg/ml is shown in Fig. 4.

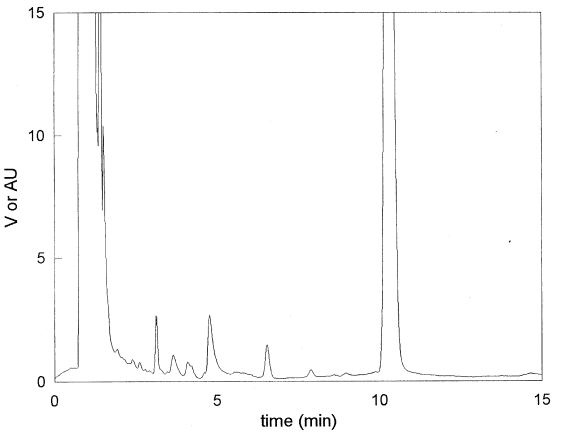

Fig. 4. Chromatogram typical for the analysis of efavirenz: a patient sample containing 7.0 μg/ml.

Table 3
Stability of efavirenz in spiked human plasma samples

Storage condition	Concentration (ng/ml)	Recovery (%)	RSD[a] (%)	n[b]
1 h at 60°C	251	93.4	2.3	6
	1002	94.3	0.7	6
	4008	98.6	4.6	6
	8015	104.9	4.6	6
24 h at 25°C	251	98.3	3.7	6
	1002	97.7	2.1	6
	4008	100.7	1.2	6
	8015	109.5	3.1	6
7 days at 4°C	251	94.7	3.2	6
	1002	98.3	0.8	6
	4008	100.0	1.1	6
	8015	109.4	3.7	6
30 days at −30°C	251	95.4	1.5	6
	1002	97.2	1.1	6
	4008	100.4	2.0	6
	8015	105.9	2.4	6
Three freeze–thaw cycles	251	95.7	3.1	6
	1002	99.3	2.1	6
	4008	101.7	1.7	6
	8015	105.9	2.4	6

[a] RSD=Relative standard deviation.
[b] n=Number of replicates.

4. Conclusion

In conclusion, a rapid, sensitive, specific and validated assay for the quantitative determination of efavirenz in human plasma is described. The applicability of the assay for pharmacokinetic research in HIV-1-infected individuals is demonstrated with the analysis of plasma samples from HIV-1-infected patients at steady-state. The assay meets all current requirements for the validation of a bioanalytical method. This HPLC assay can be used for pharmacokinetic studies with efavirenz in HIV-1-infected individuals and can readily be used to monitor efavirenz plasma concentrations in a hospital laboratory.

Acknowledgements

The authors would like to thank The Du Pont Merck Pharmaceutical Company, USA, and Merck Sharp&Dohme, The Netherlands, for supplying the efavirenz reference compound.

References

[1] J.C. Adkins, S. Noble, Drugs 56 (1998) 1055.
[2] Du Pont Pharmaceuticals Company, Sustiva (efavirenz capsules), prescribing information, 17 Sept. 1998; http://www.sustiva.com
[3] L. Bacheler, O. Weislow, S. Snyder, G. Hanna, in: 12th World AIDS Conference, Geneva, 1998, Abstract 41213.
[4] D. Mayers, J. Jemsek, E. Eyster, K. Tashima, M. Thompson, N. Ruiz, in: 12th World AIDS Conference, Geneva, 1998, Abstract 22340.
[5] S. Riddler, J. Kahn, C. Hicks, D. Stein, J. Horton, N. Ruiz, in: 12th World AIDS Conference, Geneva, 1998, Abstract 12359.
[6] D. Havlir, C. Hicks, J. Kahn, S. Riddler, Q. Lino, N. Ruiz, in: 38th Interscience Conference on Antimicrobal Agents and Chemotherapy, San Diego, CA, 1998, Abstract I-104.
[7] J. Morales-Ramirez, K. Tashima, D. Hardy, P. Johnson, M. Nelson, S. Staszewski, D. Farina, N. Ruiz, in: 38th Interscience Conference on Antimicrobal Agents and Chemotherapy, San Diego, CA, 1998, Abstract I-103.
[8] H.T. Karnes, C. March, J. Pharm. Biomed. Anal. 9 (1991) 911.
[9] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, in: Chemometrics – A Textbook, Elsevier, New York, 1988, p. 84.
[10] R.P.G. van Heeswijk, R.M.W. Hoetelmans, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 713 (1998) 395.