ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 925–934

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection

C.Z. Matthews [a], E.J. Woolf [a,*], R.S. Mazenko [a], H. Haddix-Wiener [a], C.M. Chavez-Eng [a], M.L. Constanzer [a], G.A. Doss [b], B.K. Matuszewski [a]

[a] *Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA*
[b] *Department of Drug Metabolism, Merck Research Laboratories, Rahway, NJ 07065, USA*

Received 28 September 2001; accepted 12 November 2001

Abstract

Methods for the quantitative determination of efavirenz in human plasma and the qualitative assessment of the stereochemical integrity of efavirenz in post-dose human plasma samples are described. After the addition of an internal standard, plasma samples were extracted with hexane–methylene chloride (65/35, v/v%). The extracts were evaporated to dryness and reconstituted in mobile phase. Upon exposure to UV light, the analyte was found to form fluorescent products; the major fluorescent product was isolated and identified as a substituted quinoline. Thus, the plasma extracts were analyzed via HPLC with post-column photochemical derivatization and fluorescence detection. Reverse phase chromatography was used for the quantitative assay, whereas chromatography with a column containing a chiral stationary phase (dinitrobenzoyl leucine) was used for the stereochemical assessment. The quantitative assay has been validated in the concentration range of 50–1000 ng/ml using 0.5 ml samples. Analyte recovery was better than 89% at all points on the standard curve. Intra-day precision was better than 5% C.V., while accuracy was between 95 and 104% of nominal over the range of the assay. The selective detection method reduces the likelihood of interference by co-administered medications or endogenous species. The stereochemical configuration of efavirenz was confirmed to remain intact in post-dose human plasma samples. The quantitative method has been successfully utilized to support a study in which a possible drug interaction between co-administered HIV protease inhibitors and efavirenz was evaluated.

Keywords: Efavirenz; Analysis; Plasma; Fluorescence; Photochemistry; Stereochemistry; HPLC

* Corresponding author. Tel.: +1-215-652-5559; fax: +1-215-652-8548.
E-mail address: woolf@merck.com (E.J. Woolf).

0731-7085/02/$ - see front matter
PII: S0731-7085(01)00709-9

1. Introduction

Efavirenz (Fig. 1) is a specific, non-nucleoside reverse transcriptase inhibitor (NNRTI) of human immunodeficiency virus type 1 (HIV-1) [1]. The compound contains one chiral center whose absolute configuration is *S*. The drug is commonly prescribed in combination with other anti-viral agents for the treatment of acquired immune deficiency syndrome (AIDS) [2]. The routine use of efavirenz in combination with other drugs necessitates that assays for its determination in human plasma, used to support pharmacokinetic drug-interaction studies, be highly selective.

Several assays for the determination of efavirenz in human plasma, either alone [3,4] or in combination with other retroviral agents [5–7], have recently appeared in the literature. Each of the assays, however, has drawbacks which limit their use for the analysis of samples from human pharmacokinetic studies designed to study drug–drug interactions. These limitations include run times of greater than 30 min [5–7], a complex sample preparation procedure requiring an initial liquid–liquid extraction and a hexane wash of the reconstituted sample [3] and lack of a suitable internal standard for the assay [4].

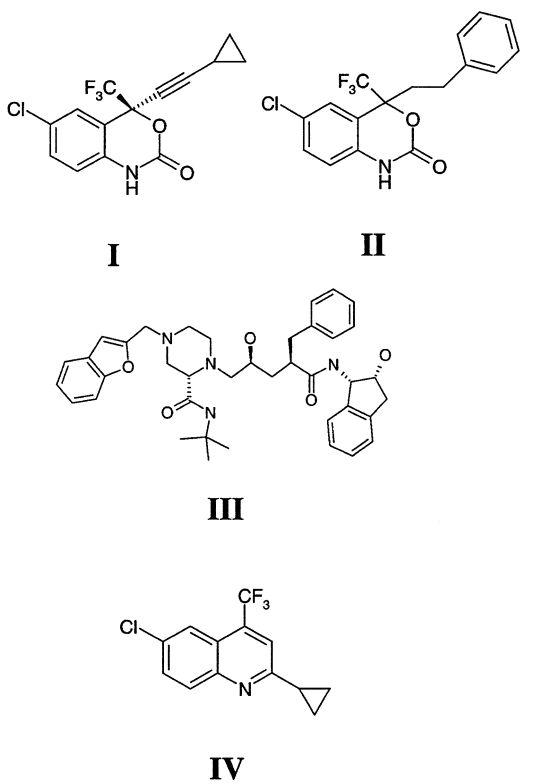

Fig. 1. Structures of efavirenz (**I**), internal standard (**II**), the experimental protease inhibitor (**III**) and the major photolysis product of efavirenz (**IV**).

An assay for the determination of efavirenz in plasma was required to support a clinical study designed to assess the effect of the co-administration of indinavir and an experimental protease inhibitor, *N*-(2(*R*)-hydroxy-1(*S*)-indanyl)-2(*R*)-phenylmethyl-4(*S*)-hydroxy-5-{1-[4-(2-benzo[*b*]furanylmethyl)-2(*S*)-*N*′-(*tert*-butyl-carboxamido)-piperazinyl]}-pentaneamide (compound **III**) [8,9], on the pharmacokinetics of efavirenz. While the use of LC with tandem mass spectrometric detection (HPLC-MS/MS) for such an assay is relatively common, we desired that the assay for efavirenz be accessable to laboratories, for example, in hospitals or clinics, that do not have access to such instrumentation, hence, the option of using HPLC-MS/MS for the assay was eliminated from consideration. Additionally, in anticipation of potential questions from regulatory agencies, it was desired to identify conditions that would enable the stereochemical integrity of efavirenz to be determined in post-dose plasma samples.

During initial experiments it was discovered that efavirenz forms fluorescent species following exposure to UV light. This discovery opened the possibility of using HPLC in combination with post-column photochemical derivatization and fluorescence detection as the basis for highly selective assays for efavirenz in human plasma. We have successfully used this technique in our laboratories for the determination of the cyclooxygenase-2 inhibitors rofecoxib [10] and etoricoxib [11]. Experiments pertaining to the isolation and characterization of the major photolysis product of efavirenz, development of chromatographic conditions to separate efavirenz from its *R* enantiomer to enable a qualitative assessment of the stereochemical configuration of efavirenz in post-dose plasma samples, as well as details of an assay for the quantitative determination of efavirenz in human plasma using a simple one step liquid–liquid extraction for analyte isolation followed by HPLC with post-column photochemical derivatization and fluorescence detection are described in this publication.

2. Experimental

2.1. Materials

Efavirenz (**I**) and the internal standard, **II** (Fig. 1) were provided by the DuPont-Merck Pharmaceutical Co. (now DuPont Pharmaceuticals, Newark, DE, USA). A racemic mixture of efavirenz and its *R* enantiomer as well as a pure sample of the *R* enantiomer was obtained from the Medicinal Chemistry Department of Merck Research Laboratories. Acetonitrile (ACN), hexane and methylene chloride (Omnisolve HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Ammonium hydroxide and isopropanol were also obtained from EM Science (Gibbstown, NJ, USA). All other reagents were ACS grade from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified with a Milli-Q system and had a resistivity of 18.2 MΩ cm at the outlet. Drug free human control plasma (EDTA as anti-coagulant) was purchased from Biological Specialty Corp. (Colmar, PA, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin–Elmer (Norwalk, CT, USA) Model 410 pump, a WISP 715 automatic injector (Waters Assoc., Milford, MA, USA), a Jones Chromatography (Lakewood, CO, USA) column heater, an AURA Industries (Staten Island, NY, USA) photochemical reactor consisting of a 254 nm UV lamp mounted with a 10.0 m, 0.3 mm ID reaction coil and a Perkin–Elmer model LC 240 fluorescence detector (Norwalk, CT, USA). The photochemical reactor was installed between the analytical column and the fluorescence detector. A block diagram of the HPLC system is shown in Fig. 2. The detector output was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 941 analog-to-digital interface.

Absorption and fluorescence spectra were obtained using a diode array spectrophotometer (HP 8452, Hewlett–Packard, Palo Alto, CA, USA) and an Hitachi (Danbury, CT, USA) Model F-4500 spectrofluorometer, respectively.

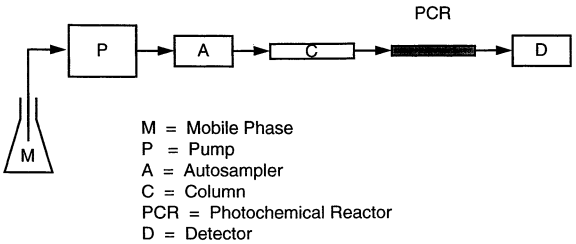

Fig. 2. Scheme of HPLC and post-column photochemical reactor system.

NMR spectra were obtained with a Varian (Palo Alto, CA, USA) UNITY 500 MHz instrument using $CDCl_3$ as solvent.

Off-line irradiations of samples were performed using a 'merry-go-round' photochemical reactor (Rayonet, Hamden, CT, USA) equipped with four 300 nm lamps.

An API III+ triple quadrupole mass spectrometer (PE-Sciex, Thronhill, Canada) was used to obtain mass spectra of the major photolysis product of efavirenz. The mass spectrometer was operated in the positive-ion mode at unit mass resolution. Both heated nebulizer atmospheric pressure chemical ionization and ion-spray interfaces were utilized.

2.3. Preparation and isolation of the major photolysis product of efavirenz

Ninety milliliters of a 1 mg/ml solution of efavirenz in ACN were divided between six quartz glass tubes. The tubes were then simultaneously exposed to 300 nm light in the photochemical reactor for 30 min. Reverse phase HPLC analysis (150×4.6 mm BDS Hypersil C18 analytical column, 60/40 v/v% ACN/20 mM Tris buffer (pH 7.4) at 1.2 ml/min with UV detection at 248 nm) indicated that approximately 40% of the efavirenz had been converted to products.

The mixture after photolysis was evaporated to dryness under nitrogen to yield 90.3 mg of a dark brown oil. The oil was dissolved in 1 ml of benzene. The benzene mixture was then applied to a column (10×220 mm) packed with silica gel (70–230 mesh). The column was eluted sequen-

tially with four 10 ml fractions of the following solvents: hexane, 1, 2, 4, 6, 8, 10, 15, 25, and 50% diethyl ether in hexane, and diethyl ether. Finally, the column was washed with two 10 ml portions each of ethyl acetate and methanol. Each fraction ($n = 48$) was evaporated to dryness, reconstituted with 500 μl of ACN and analyzed via HPLC using the system described above. The major product (11.5 mg isolated) was eluted in fractions 9 and 10.

2.4. Chromatographic conditions for the quantitative efavirenz plasma assay

The HPLC mobile phase for the quantitative assay consisted of a mixture of ACN and phosphate solution (53/47, v/v%). The phosphate solution (50 mM, pH 3.5) was prepared by mixing 1.9 l of deionized water and 6.8 ml of 85% phosphoric acid, adjusting the pH of the mixture to 3.5 with 30% ammonium hydroxide, transferring the solution to a 2 l volumetric flask and adding deionized water to the mark. The mobile phase was filtered through a nylon filter (0.45 μm) prior to use. A flow rate of 1.0 ml/min was used to deliver the mobile phase through a YMC (Wilmington, NC, USA) Octyl S-5 120 A (23 × 4 mm) guard column connected to a YMC-Pack C8 analytical column (100 × 4.6 mm, S-5 micron, 120 A). The temperature of the columns was maintained at 30 °C. The fluorescence detector was set at an excitation wavelength of 310 nm. The emission wavelength was controlled via a 390 nm long pass filter. Sample injection volume was 50 μl.

2.5. Chromatographic conditions for the chiral separation of efavirenz and its enantiomer

Efavirenz and its enantiomer were separated on a dinitrobenzoyl (DNB) leucine column (4.6 × 250 mm, J.T. Baker, Phillipsburg, NJ, USA) using a mobile phase of 2.5% ethanol in hexane at a flow rate of 1.2 ml/min. The column was maintained at 45 °C. Detection conditions were identical to those described above for the quantitative assay. Sample injection volume was 25 μl.

2.6. Preparation of standards

A 50 μg/ml stock solution of efavirenz was prepared by weighing 2.5 mg of reference material into a 50-ml red volumetric flask, dissolving the compound in 25 ml of ACN, and filling the flask to volume with water. Working standards at concentrations of 20 and 15 μg/ml were prepared by dilution of the 50 μg/ml stock solution with ACN–water (50/50, v/v%). Working standards at concentrations of 10, 8, 4, 2 and 1 μg/ml were prepared by dilution of the 20 μg/ml working standard with ACN–water (50/50, v/v%).

Analysis standards were prepared by spiking 25 μl of each working standard to 0.5 ml of drug-free human control plasma (EDTA as anti-coagulant). These standards were used to analyze samples containing efavirenz over the range of 50–1000 ng/ml. Clinical samples containing higher concentrations of efavirenz were diluted with control plasma prior to analysis. Working standards solutions were found to be stable for at least 1 month when they were stored at room temperature and protected from light.

Quality control (QC) samples containing efavirenz at concentrations of 750 ng/ml (High QC), 400 ng/ml (Medium QC) and 100 ng/ml (Low QC) were prepared by dilution of 1-ml aliquots of solutions of efavirenz in 50/50 ACN–water (50/50, v/v%) at concentrations of 75, 40 and 10 μg/ml to 100 ml with control plasma. The stock solution from which the QC spiking solutions were diluted was prepared from a separate weighing of reference standard from that used to prepare the working standards described above. The QC samples were divided into 1-ml aliquots and stored at −20 °C.

2.7. Extraction of human plasma

Aliquots (0.5 ml) of clinical samples were pipetted into 15-ml screw cap disposable glass centrifuge tubes (Fisher Scientific, Fair Lawn, NJ, USA). A 25 μl aliquot of ACN–water (50/50, v/v%) was added to each of the clinical samples to make their volume equal to those of the standards. A 25 μl volume of internal standard (**II**) solution (10 μg/ml in 50/50 ACN–water, v/v%)

was added to the samples and standards and the tube contents were vortex mixed. Eight milliliters of extraction solvent (35/65, v/v% methylene chloride–hexane) were added to the tubes. The centrifuge tubes were capped, shaken on a flat bed shaker for 15 min, and centrifuged ($2060 \times g$) for 5 min to separate the phases. The tubes were then placed in an isopropanol/dry ice bath for 5 min to freeze the lower aqueous layer. The upper layer was decanted into a clean culture tube (13×100 mm) and the extraction solvent was evaporated with nitrogen using a Turbovap LV evaporator (Zymark, Hopkinton, MA, USA) set at 40 °C for 20 min. The extract residues were reconstituted in 500 μl of HPLC mobile phase. The reconstituted extracts were finally transferred into autosampler vials prior to injection into either the chiral or quantitative assay HPLC systems.

All sample preparation and analysis procedures were conducted under yellow (UV free) lighting to eliminate possible analyte photolysis during sample processing.

3. Results and discussion

3.1. Spectroscopic characterization of efavirenz

UV spectra of efavirenz obtained in 50/50 v/v% ACN/20 mM phosphate buffer exhibited three maxima at wavelengths of 206 ($\varepsilon = 32\,000$/M cm), 248 ($\varepsilon = 14\,600$/M cm) and 292 nm ($\varepsilon = 1950$/M cm). The maxima and molar extinction coefficients did not vary significantly over the pH range of 3.0–7.4. The compound was not found to be fluorescent.

Irradiation of solutions of efavirenz in ACN with 300 nm UV light caused significant changes in the UV spectra of the compound. The intensity of the 248 nm band increased slightly, the 292 nm band broadened significantly and a band with a maximum at 400 nm appeared. The 400 nm band was found to disappear upon further irradiation of the solution. Additionally, after irradiation, solutions were found to be fluorescent, with an emission maxima at 390 nm.

HPLC analysis of the irradiated solutions showed a decrease in the efavirenz peak with the corresponding appearance of several peaks at retention times longer than that of efavirenz.

Based on these results, fluorescence detection following post-column photochemical derivatization was chosen as the method for detecting efavirenz in plasma extracts following HPLC separation. The use of this approach provides a higher degree of selectivity as compared to UV detection, which has been employed in all assays published to date [3–7]. Such selectivity was important given the fact that we desired to determine efavirenz in the presence of other co-administered medications, the majority of which are UV absorbing, but not fluorescent.

3.2. Structure elucidation of the major photolysis product of efavirenz

The major photolysis product of efavirenz was isolated via column chromatography following the photolysis of a 1 mg/ml solution of the compound on a 90 mg scale. The product was found to be a white solid material with a melting point of 62.5–63.5 °C.

The product was introduced to a tandem quadrupole mass spectrometer using both heated nebulizer and ion spray HPLC-MS/MS interfaces. The Q1 spectrum of the product, which was found not to be interface dependent, showed a single ion at m/z 272, 44 units less than the $M + H^+$ peak for efavirenz that was observed at m/z 316 under the same instrumental conditions. The loss of 44 amu was believed to correspond to the loss of CO_2 from the molecule, indicating that the product was probably formed through the photodecarboxylation of efavirenz. The collision induced dissociation spectrum of the ion at m/z 272 gave major fragments at 244, 230, 224, 210, 202, 189, 175, 167 and 140.

The propensity of the loss of 44 amu from efavirenz was clearly demonstrated by the presence of a very strong fragment at m/z 272 that was observed in the Q1 APCI spectrum of the parent compound when it was introduced to the mass spectrometer via a heated nebulizer; apparently thermal decarboxylation occurred within the mass spectrometer interface under these conditions. This fragment was absent in the Q1 spec-

trum when efavirenz was introduced to the mass spectrometer via an ion–spray interface where no heat was applied to the sample during the ionization process.

High resolution FAB-MS of the product indicated that the exact mass of the major photolysis product was 272.045543. The most likely molecular formula corresponding to this mass was calculated to be $C_{13}H_{10}NClF_3$.

High resolution NMR of the major photolysis confirmed the structure as 6-chloroquinoline substituted in positions 2 and 4 with cyclopropyl and trifluoromethyl groups, respectively (Compound **IV**, Fig. 1). The molecular formula of this compound is consistent with that suggested by the high resolution FAB-MS. Compound **IV** has been previously identified as an undesired product that was isolated during the development of the efavirenz synthetic process [12].

Attempts to isolate and identify the minor photolysis products observed in the chromatogram of the irradiated solution failed, most likely due to the chemically labile nature of these compounds.

3.3. Extraction procedure development

Protein precipitation [4], liquid–liquid extraction [3,6], and solid phase extraction [5,7] have been used to isolate efavirenz from human plasma. The compound was found to readily extract into either methyl-*t*-butyl ether or mixtures of methylene chloride and hexane. The analyte could also be extracted using solid phase extraction in the 96 well format with a plate containing C8 sorbent disks. Extracts prepared using solid phase extraction were found to contain endogeneous peaks which interferred with the quantitation of efavirenz; these peaks were absent in samples prepared using liquid–liquid extraction, hence the liquid–liquid extraction procedure was further optimized.

Optimum recoveries were obtained using a 65/35 v/v% mixture of hexane–methylene chloride. Additionally, attempts to buffer plasma samples prior to extraction introduced additional endogenous peaks into the chromatograms of plasma extracts without affecting the recovery of efavirenz or internal standard. Hence, no buffer was added to plasma samples prior to extraction.

3.4. Extraction recovery

The recovery of the extraction procedure was determined by comparing the responses (peak area) of the working standards of efavirenz and internal standard injected directly into the HPLC system with those of extracted plasma standards. The results (Table 1) indicate that the recovery of efavirenz from human plasma was greater 89% at all concentrations tested. The high extraction recovery of efavirenz from unbuffered plasma can be attributed to the fact that efavirenz is not in an ionized form under physiological conditions [13]; the neutral molecule would be expected to extract with good efficiency into organic solvents. Recovery of internal standard from human plasma was greater than 92% at a concentration of 500 ng/ml.

3.5. Assay selectivity

Fig. 3 shows chromatograms of extracted control plasma, a plasma standard containing efavirenz (50 ng/ml) and internal standard (500 ng/ml), and a post-dose plasma sample from a subject following a 600 mg dose of efavirenz. A comparison of Fig. 3A with B illustrates that no endogenous peaks co-elute with either the analyte or internal standard.

Fig. 4A shows a chromatogram of a plasma sample from subject following the administration of indinavir and the experimental protease inhibitor, **III** [8,9]. Fig. 4B shows a chromatogram of a plasma sample from a subject following the administration of indinavir, **III** and efavirenz. A

Table 1
Extraction recovery of efavirenz from human plasma

Nominal concentration (ng/ml)	Extraction recovery (%)	C.V.[a] (%)
50	99.0	6.7
100	89.3	4.1
200	91.9	1.5
400	96.4	2.1
500	97.6	2.1
750	95.1	1.2
1000	92.9	4.6

[a] Coefficient of variation, $n = 5$.

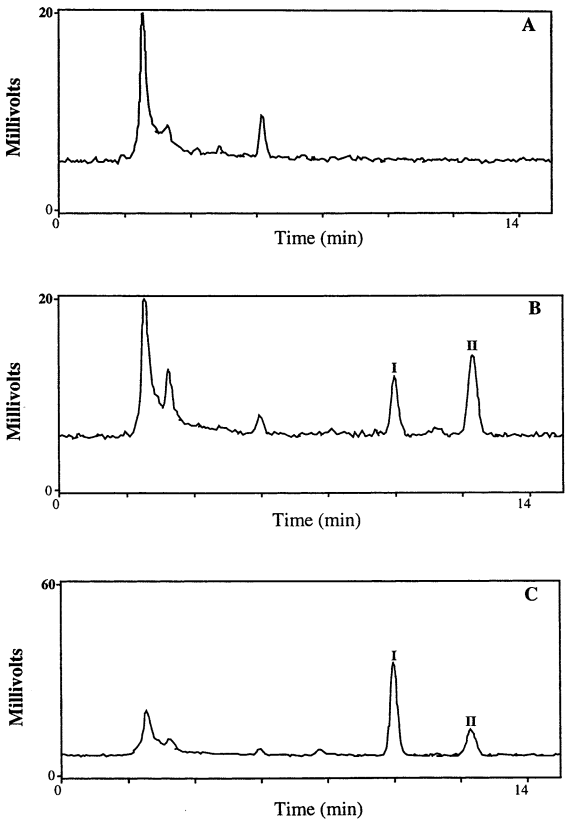

Fig. 3. Representative chromatograms of plasma samples. (A) Control human plasma; (B) plasma spiked with 50 ng/ml efavirenz (**I**) and internal standard (**II**, 500 ng/ml); (C) post-dose plasma sample from a human subject obtained after the administration of efavirenz. The concentration of efavirenz is equivalent to 1100.9 ng/ml (sample diluted 1:5 before analysis).

comparison of Fig. 4A and B indicates that neither indinavir, **III** nor their respective metabolites interfered with the analysis of efavirenz, thus illustrating the selectivity of the method in the presence of these co-administered compounds.

3.6. Assay linearity

Several linear regression models (non-weighted, $1/x$ weighting and $1/y$ weighting) were evaluated with respect to fitting the standard curve. Weighted (weighting factor $= 1/y$ where $y =$ peak height ratio) least-squares regression calibration curves, constructed by plotting the peak height ratio of efavirenz to internal standard versus standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration ranges of the plasma assay. Choice of the $1/y$ weighted least-squares regression was based on the fact that use of this model resulted in less than a 6% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equation.

3.7. Assay precision and accuracy

Replicate standards ($n = 5$) were analyzed to assess the within-day variability of the assays. The mean assayed concentrations as well as the mean accuracy and precision of the analyses, obtained using the human plasma assay, are shown in Table 2.

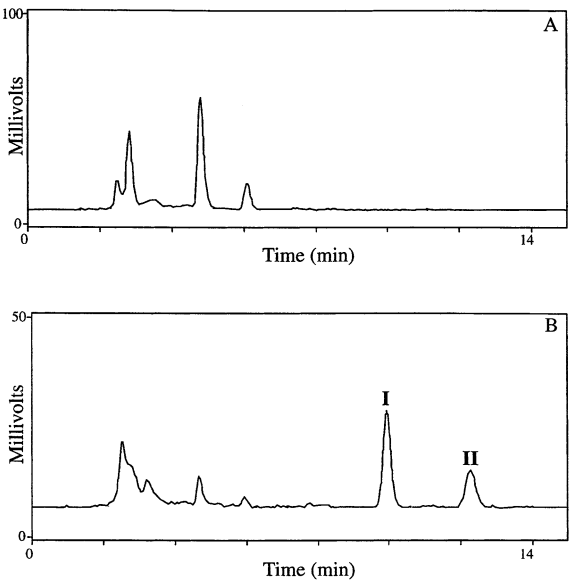

Fig. 4. Representative chromatograms of plasma samples from efavirenz, indinavir, and compound **III** interaction study. (A) Post-dose sample from a subject administered indinavir and **III** only; (B) post-dose sample from a subject administered indinavir, **III** and efavirenz. The concentration of efavirenz is equivalent to 1670.2 ng/ml (sample diluted 1:10 before analysis).

Table 2
Intra-day precision and accuracy of the efavirenz assay as assessed by the replicate ($n = 5$) analysis of standards

Nominal standard concentration (ng/ml)	Mean ($n = 5$) analyzed standard concentration (ng/ml)	Accuracy[a] (%)	C.V.[b] (%)
50	51.5	103.0	4.8
100	95.1	95.1	3.2
200	195.4	97.7	3.7
400	394.2	98.5	1.6
500	501.3	100.3	2.4
750	781.0	104.1	3.1
1000	1038.4	103.8	2.1

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

3.8. Quality control samples

Quality control samples (QCs), containing concentrations of 100, 400 and 750 ng/ml of efavirenz in plasma were prepared and frozen (− 20 °C) in 1 ml volumes. It was found that efavirenz in QC human plasma samples was stable through three freeze-thaw cycles. The QC samples were analyzed each day along with human clinical samples from clinical studies to assess the inter-day variability of the assay. Representative plasma QC sample data acquired over a 2-week period during the analysis of clinical samples is shown in Table 3. The accuracy (within 10% of nominal at all concentrations) and precision (C.V. under 5% at all concentrations) of this data are well within the guidelines of bioanalytical method validation [14].

3.9. Limit of quantification

The limit of quantification of the assays, defined as the lowest concentration that yielded a within-day coefficient of variation (C.V.) of less than 10% and an within-day accuracy between 90 and 110% of nominal concentration, was 50 ng/ml in human plasma. This limit of quantification was achieved by injecting only 10% of the reconstituted sample. In theory, a lower limit of quantification could be achieved by increasing the percentage of the extract injected, through either an increase in injection volume or a decrease in the volume of mobile phase in which the sample was reconstituted. The limit of 50 ng/ml was, however, found to be sufficient to support pharmacokinetic studies of efavirenz at its recommended clinical dose.

3.10. Chiral separation development

The molecule of efavirenz contains one chiral center whose absolute configuration is *S*. While the in vivo inversion of configuration at this center was believed to be unlikely, it was desirable to confirm it experimentally. Verification required the development of a chromatographic method capable of separating efavirenz from its *R* enantiomer. Baseline separation of efavirenz from its *R* isomer was achieved using either a dinitrobenzoyl (DNB) leucine column or a *S*,*S* Whelk-o column with a mobile phase of 2.5% ethanol in hexane. Only partial separation of the isomers was ob-

Table 3
Inter-day variability of the assays for determination of efavirenz as assessed by the coefficient of variation of low, medium and high QC samples

Nominal Q.C. concentration (ng/ml)	Mean* analyzed concentration (ng/ml)	C.V.[a] (%)
100	90.3	4.1
400	367.9	2.6
750	722.8	2.7

[a] $n = 5$ Runs over a 2 week period.

served on a DNB-glycine column under the same chromatographic conditions. Additional experiments indicated that the enantiomer peaks were more symmetrical on the DNB-leucine column. Increasing column temperature from 25 to 45 °C on the DNB-leucine column was found to further sharpen peaks and decrease retention while maintaining baseline separation of the stereoisomers, hence, these conditions were used for the analysis of post-dose plasma samples.

3.11. Assessment of the stereochemical integrity of efavirenz in post-dose human plasma samples

Fig. 5A shows a chromatogram of an extract of control plasma obtained under the conditions of the chiral separation. A chromatogram of an extract of a plasma sample spiked with 2500 ng/ml of a racemic mixture of efavirenz and its *R* enantiomer is shown in Fig. 5B. A comparison of Fig. 5A with B indicates that no endogenous components are detectable at the retention times of efavirenz or its enantiomer. The lack of endogenous interferences under these conditions is most likely due to the selective nature of the post-column photochemical derivatization/fluorescence detection scheme.

A chromatogram of an extract of a plasma sample obtained following the oral administration of a dose of efavirenz is shown in Fig. 5C. Only efavirenz is detectable in this sample, the *R* enantiomer was not observed in this, or any other post-dose plasma samples that were analyzed. This data indicates that the stereochemical integrity of efavirenz remains intact following oral administration.

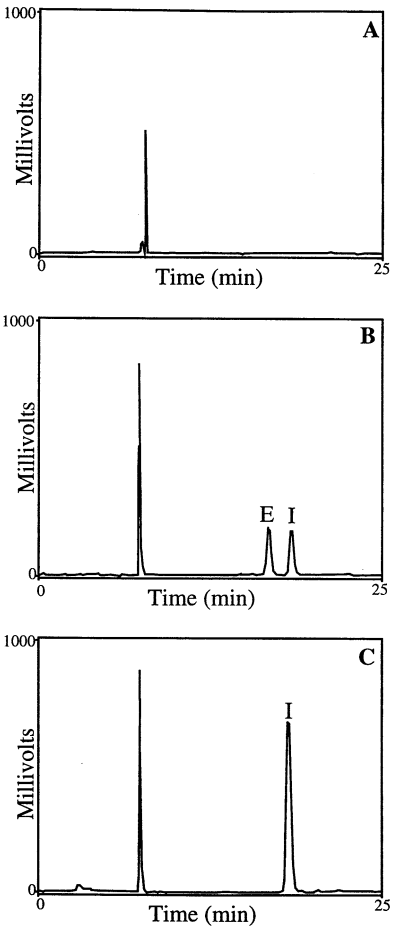

Fig. 5. Representative chromatograms obtained under the conditions used to assess the stereochemical integrity of efavirenz in post-dose plasma samples. (A) Control plasma; (B) plasma spiked with 2500 ng/ml of a racemic mixture of efavirenz (**I**) and its enantiomer (**E**); and (C) post-dose sample from a subject administered efavirenz.

3.12. Quantitative clinical sample analysis

Plasma samples from a clinical study designed to evaluate the effect of the co-administration of the HIV protease inhibitors indinavir and **III** on efavirenz plasma concentrations were analyzed using the described assay. During the first period of this study, subjects were administered 600 mg of efavirenz once daily in the evening for 7 days. Plasma samples for efavirenz analysis were collected on day 7. During the second period of the study, subjects received 1600 mg **III** and 800 mg indinavir once daily in the morning and 600 mg efavirenz once daily in the evening for 7 days. Plasma samples for efavirenz analysis were again collected on the 7th day of dosing. An efavirenz concentration versus time profile from a subject participating in this study is shown in Fig. 6. The co-administration of indinavir and **III** appears to have a modest effect on efavirenz plasma concentrations. The analysis of plasma samples from this

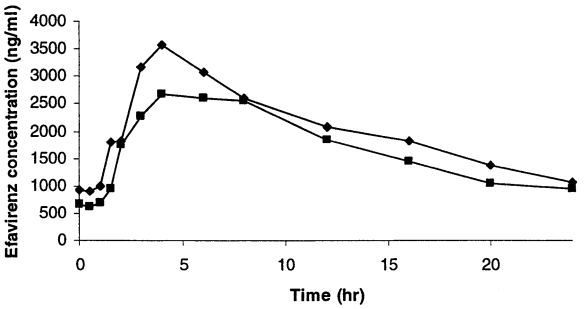

Fig. 6. Efavirenz plasma concentration versus time profile from a subject following 7 days of administration of efavirenz (◆) or efavirenz together with the HIV protease inhibitors indinavir and **III** (■).

study demonstrates the applicability of the assay to the analysis of clinical samples.

4. Conclusions

A selective assay for determination of efavirenz in human plasma using HPLC with fluorescence detection following post-column photochemical derivatization has been developed and validated. The assay has been found to be precise, accurate and suitable for the analysis of plasma samples collected during clinical studies.

Conditions using the post-column photochemical derivatization/fluorescence detection scheme along with chromatography on a chiral stationary phase were also developed to assess the stereochemical integrity of efavirenz in post-dose human plasma samples. The stereochemical configuration of efavirenz was verified to remain intact in post-dose plasma samples.

The use of the selective detection technique was found to significantly reduce the possibility of interference from endogenous compounds and co-administered medications under the conditions of both the quantitative assay and the stereochemical assessment.

Acknowledgements

The authors would like to thank H. Ramjit for obtaining the FAB-MS of the major photolysis product of efavirenz and S. Young for providing the samples of the racemic mixture of efavirenz and its *R* enantiomer as well as a pure sample of the *R* enantiomer. The human plasma samples were obtained from clinical studies directed by Dr William Ju, Department of Clinical Pharmacology, Merck Research Laboratories.

References

[1] S.D. Young, S.F. Britcher, L.O. Tran, L.S. Payne, W.C. Lumma, T.A. Lyle, J.R. Huff, P.S. Anderson, D.B. Olsen, S.S. Carroll, D.J. Pettibone, J.A. O'Brien, R.G. Ball, S.K. Balani, J.H. Lin, I.W. Chen, W.A. Schleif, V.V. Sardana, W.J. Long, V.W. Byrnes, E.A. Emini, Antimicrob. Agents Chemother. 39 (1995) 2602–2605.
[2] J.C. Adkins, S. Noble, Drugs 56 (1998) 1055–1064.
[3] P. Villani, M. Pregnolato, S. Banfo, M. Rettani, D. Burroni, E. Seminari, R. Maserati, M.B. Regazzi, Ther. Drug Monitoring 21 (1999) 346–350.
[4] A.I. Veldkamp, R.P.G. van Heeswijk, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, R.M.W. Hoetelmans, J. Chromatogr. B 734 (1999) 55–61.
[5] C. Marzolini, A. Telenti, T. Buclin, J. Biollaz, L.A. Decosterd, J. Chromatogr. B 740 (2000) 43–58.
[6] V. Proust, K. Toth, A. Hulin, A.M. Taburet, F. Gimenez, E. Singlas, J. Chromatogr. B 742 (2000) 453–458.
[7] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B 744 (2000) 227–240.
[8] M.J. Rose, S.A. Merschman, E.J. Woolf, B.K. Matuszewski, J. Chromatogr. B 732 (1999) 425–435.
[9] M.J. Rose, S.A. Merschman, R. Eisenhandler, E.J. Woolf, K.C. Yeh, W. Fang, L. Lin, J. Hsieh, M.P. Braun, G.J. Gatto, B.K. Matuszewski, J. Pharm. Biomed. Anal. 24 (2000) 291–305.
[10] E. Woolf, I. Fu, B. Matuszewski, J. Chromatogr. B 730 (1999) 221–227.
[11] C.Z. Matthews, E.J. Woolf, L. Lin, W. Fang, J. Hsieh, S. Ha, R. Simpson, B.K. Matuszewski, J. Chromatogr. B 751 (2001) 237–246.
[12] C.Y. Chen, L. Tan, Enantiomer 4 (1999) 599–608.
[13] S.R. Rabel, M.B. Maurin, S.M. Rowe, M. Hussain, Pharm. Dev. Tech. 1 (1996) 91–95.
[14] V.P. Shah, K.K. Midha, J.W.A. Findley, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patmaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pham. Res. 17 (2000) 1551–1557.