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Simple and rapid method for the simultaneous determination of the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma using liquid chromatography

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Abstract

Efavirenz and nevirapine are non-nucleoside reverse transcriptase inhibitors for the treatment of HIV-1-infected individuals. A simple and rapid high-performance liquid chromatographic method for the simultaneous quantification of efavirenz and nevirapine in human plasma suitable for therapeutic drug monitoring is described. Sample pre-treatment consisted of protein precipitation with acetonitrile and subsequently dilution with distilled water. The drugs were separated from endogenous compounds by isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection at 275 nm. The method was validated over the therapeutically relevant concentration range of 0.05–15.0 mg l^{-1} and 0.25–15.0 mg l^{-1} for efavirenz and nevirapine, respectively, using a volume of 100 μl of plasma. The calibration curves were linear over this concentration range. Carbamazepine was used as internal standard. The assay proved to be accurate (accuracies varied between −12.7 and 8.5%) and precise (intra- and inter-assay precisions were less then 5.9%). The tested batches of control human plasma and frequently co-administered drugs did not interfere with the described methodology. Efavirenz and nevirapine were stable under various relevant storage conditions. This validated assay is suited for use in pharmacokinetic studies with efavirenz and nevirapine and can readily be implemented in the setting of a hospital laboratory for the monitoring of efavirenz and nevirapine concentrations.

Keywords: Efavirenz; Nevirapine

1. Introduction

Efavirenz and nevirapine are non-nucleoside reverse transcriptase inhibitors (NNRTIs). NNRTIs are one of the three classes of drugs currently used to treat human immunodeficiency virus (HIV) infection. These drugs inhibit non-competitively the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). NNRTIs bind directly and reversibly to the catalytic site of the reverse transcriptase enzyme and therefore, interfere with viral RNA to DNA-directed polymerase activities [1,2].

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Relationships between plasma drug concentrations of NNRTIs and efficacy and toxicity have been identified [3,4]. The plasma concentration of the NNRTIs depends on the pharmacokinetic parameters, which can be influenced by patient characteristics (i.e. co-morbidity, demographics). Large inter-patient pharmacokinetic variability suggest a role for therapeutic drug monitoring to individualise antiretroviral therapy, when target values of the NNRTIs have been established. There is an urgent need for a simple and rapid assay for routine measurement of NNRTI concentrations in plasma and large-scale pharmacokinetic studies. Ideally, micro-volumes of plasma are required for this assay, which enables studies in special populations like HIV-1-infected children. Moreover, sample pre-treatment should be minimal. Obviously, a combined assay of these extensively used compounds strongly reduces workload for routine measurements. Current assays, however, lack these requirements.

Recently, a few methods for the simultaneous determination of NNRTIs have been published [5–9]. Commonly, reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection or mass spectrometry (MS) is used. The methods of Rezk et al. [6] and Simon et al. [8] use 500 μl sample, and Aymard et al. [7] and Titier et al. [9] use 1 ml samples for the determination of the plasma levels. Besides, all methods use a complicated time-consuming sample pre-treatment consisting of solid-phase or liquid–liquid extraction combined with evaporation of the extract to dryness, which is reconstituted afterwards. Furthermore, complicated instrument set-up is necessary consisting of a combination of two HPLC systems or gradient elution [5–8]. We here report the development and validation, according to current FDA guidelines, of a simple and rapid, isocratic HPLC assay with UV detection for the simultaneous, quantitative determination of efavirenz and nevirapine in human plasma. Only 100 μl of sample was used combined with a very rapid and simple sample pre-treatment. The proposed technique implies protein precipitation, isocratic elution on a reversed-phase HPLC-system, and UV detection at a single wavelength. The usefulness of the method is demonstrated by the analysis of plasma samples of treated HIV-1-infected patients.

2. Experimental

2.1. Equipment and supplies

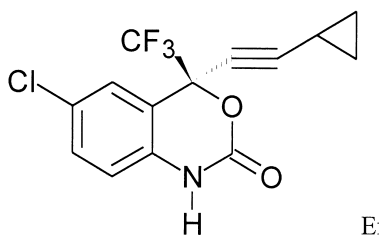
The HPLC system consisted of a P1000 solvent delivery pump (Thermo Separation Products, Fremont, CA), an SCM1000 degasser (Thermo Separation Products), an AS3000 automatic sample injection device (Thermo Separation Products), and a UV1000 wavelength detector (Thermo Separation Products). The analytical column was a Zorbax Extend C18 (150×2.1 mm I.D., 5 μm particle size; Agilent Technologies, Amstelveen, The Netherlands) protected by a ChromGuard HPLC pre-column (10× 3.0 mm I.D. reversed-phase; Varian, Middelburg, The Netherlands) and a precolumn microfilter frit, 0.5 μ*M*, C-425X (Upchurch Scientific, Oak Harbor, WA, USA). Analytical runs were processed by Chromquest on Windows (version 2.51, ThermoQuest Corporation, San Jose, USA).

2.2. Chemicals

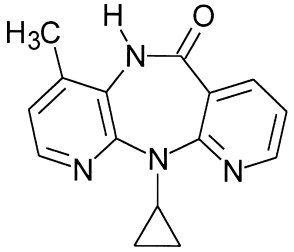
Efavirenz was kindly provided by Merck Sharp and Dohme, Haarlem, The Netherlands and nevirapine by Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA. Carbamazepine (BUFA Pharmaceutical Products, Uitgeest, The Netherlands) was used as the internal standard for this assay. Chemical structures of efavirenz, nevirapine and carbamazepine are shown in Fig. 1. Acetonitrile and methanol (both HPLC supra-gradient) were purchased from Biosolve (Amsterdam, The Netherlands). Dimethylsulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany) and triethylamine was obtained from Merck (Schuchardt, Hohenbrunn, Germany). Distilled water originated from Aqua B. Braun (Melsungen, Germany). Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands). 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% methanol (final concentration 500 mg l^{-1}).

2.3. Preparation of standards

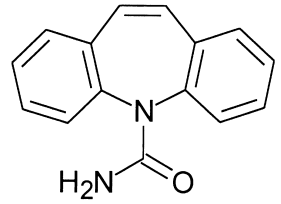
Amounts of 5 mg of the analytes were accurately



Efavirenz



Nevirapine



Carbamazepine

Fig. 1. The chemical structures of efavirenz, nevirapine and the internal standard carbamazepine.

weighted and transferred to separate volumetric flasks of 5.0 ml. Efavirenz and nevirapine were dissolved in methanol and DMSO, respectively, to give final concentrations of 1 mg ml^{-1}. Independently prepared stock solutions with separate weighting were made for the preparation of the calibration (CAL) standards and quality control (QC) samples.

The stock solutions of both efavirenz and nevirapine were used for preparing a combined CAL-working solution. This CAL-working solution was diluted with methanol to acquire CAL-working solutions at several concentration levels. Calibration concentrations of 0.05–15.0 mg l^{-1} efavirenz and nevirapine were prepared in blank human plasma. QC samples in the concentrations of 0.25, 1.0, 5.0 and 12.5 mg l^{-1} were prepared independently in a similar way. CAL and QC samples contained 5% of organic solvent.

2.4. Internal standard (IS) preparation

Carbamazepine (30 mg) was dissolved in acetonitrile to achieve a final concentration of 30 mg l^{-1} (IS-solution).

2.5. Sample pre-treatment

In an Eppendorf tube of 1.5 ml, 200 μl IS-solution was added to 100 μl of plasma. The samples were vortexed for 2 s and then mixed for 15 min on a shaking device. Afterwards the tubes were centrifuged for 10 min at 10 500 *g*, and subsequently 200 μl of the clear supernatant was transferred to another tube and mixed with 200 μl of distilled water. The tubes were mixed on a vortex for 10 s and then centrifuged for 10 min at 10 500 *g*. 200 μl of the clear supernatant was transferred to an autosampler vial.

2.6. Chromatography

The chromatographic analysis was performed at ambient temperatures on a Zorbax Extend C18 analytical column with a mobile phase composed of 25 m*M* triethylamine in water–acetonitril (65:35, v/v) pH: 11.7. Absorbance was measured at 275 nm. The flow-rate was maintained at 0.2 ml min^{-1}. Aliquots of 25 μl were injected.

2.7. Validation procedures

All validations were performed according to the recently published FDA guidelines for validation of bioanalytical assays [10].

2.7.1. Response functions

Calibration curves were constructed by least-squares linear regression analysis without weighting and by using $1/x$ and $1/x^2$ (x=concentration) as weighting factors. In order to establish the best

quantification method (with IS or without IS) and the best weighting factor, back-calculated calibration concentrations were determined. The model with the lowest total bias and the most constant bias across the concentration range was used for further analysis and quantification.

2.7.2. Accuracy and precision

Accuracy and precision were determined by analysing QC samples with analyte concentrations at the lower limit of quantification (LLQ) and in the low, mid and high concentration ranges of the calibration curves. In addition, QC samples with analyte concentrations 2.5 times higher than the upper limit of quantification (ULQ) were analysed after 5-fold dilution of the samples. Each QC sample was analysed in a minimum of five replicates together with a calibration curve, independently prepared from the control samples, in three analytical runs. The accuracy was defined as percent difference between the mean concentration and the nominal concentration. The coefficient of variation was used to report the precisions.

The intra- and inter-assay accuracies for the LLQ concentration should be within ±20% and for all other concentrations within ±15%. The precisions should be less than 20% for the LLQ and less than 15% for all other concentrations [10,11].

2.7.3. Specificity and selectivity

Six different batches of control drug-free human plasma were tested to determine whether endogenous matrix constituents co-eluted with the analytes or the internal standard (IS). Double blanks (without IS), blanks (with IS) and spiked samples (with efavirenz and nevirapine) at the LLQ were prepared, processed according to the described procedures and analysed.

To investigate the potential interference of co-medication with the quantification of the analytes, the co-medicated drugs were added to double blank samples at therapeutic drug concentrations. The samples were then processed and assayed according to the described method. The following drugs, frequently used by HIV-infected individuals, were investigated for interference: abacavir, acetaminophen, amprenavir, caffeine, delavirdine, didanosine, fluconazole, folic acid, ganciclovir, indinavir, itraconazole, lamivudine, lopinavir, methadone, nelfinavir, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampicine, ritonavir, saquinavir, stavudine, sulfamethoxazole, tenofovir, trimethoprim, zalcitabine, zidovudine and zidovudine-glucuronide. The peak areas of compounds co-eluting with one of the analytes should be less than 20% of the peak areas of the analyte at the LLQ. For compounds co-eluting with the internal standard the peak area should be less than 5% of the IS area [10].

2.7.4. Recovery

Extraction recoveries were determined by comparing the peak area of the QC samples in the low, mid and high concentrations with unprocessed solutions of corresponding concentrations in 6-fold.

2.7.5. Stability

Several stability tests were performed to verify the stability of the drugs during all handling procedures. Samples were assayed at two concentrations (at low and high concentrations) in triplicate.

The samples were kept for 24 h at ambient temperatures, 7 days at 4 °C and subjected to three freeze–thaw cycles. The concentration of the drugs after each storage period was related to the concentration of freshly prepared samples in the same analytical run. In addition, long term stability of efavirenz and nevirapine in plasma was investigated after 6 and 12 months of storage at −20 °C, respectively.

Also a stability test was performed to verify the stability of the drugs in the autosampler vials pending analysis. The samples were left at ambient temperatures in the autosampler for 5 days prior to analysis.

Furthermore, stability of the IS-solution was investigated over 24 h and 30 days at ambient temperatures. The stability of the stock solutions of efavirenz and nevirapine was determined at 24 h at ambient temperatures, and 24 and 36 months, respectively, at −20 °C.

Analytes were considered stable if the concentrations deviated less than ±20% from the concentrations of freshly prepared samples or from nominal concentrations, with coefficients of variance less than 20%. For stability of stock solutions the deviation should be less than ±5% and the C.V. values less than 5% [10].

2.8. Analysis of patient samples

Plasma samples of several HIV-infected individuals on an efavirenz or nevirapine containing regimen were analysed with the currently reported method to assess applicability of the method.

3. Results

3.1. Chromatography and detection

The starting point for the development was the previously described HPLC–UV method for efavirenz [12]. Using this system, however, nevirapine eluted without relevant retention. Because of this very short retention time, the amount of organic modifier in the mobile phase was decreased. Under these conditions efavirenz did not elute within 60 min and the chromatography of nevirapine was unacceptable. Thereupon, the pH of the mobile phase was increased to 8.5 and higher. As a consequence, a column specially designed for stable use with high pH mobile phases (Zorbax Extend), was used. Phosphate, ammonia and triethylamine buffers combined with different percentages of modifiers (both acetonitrile and methanol) were tested as mobile phase. No elution of efavirenz was obtained with methanol as modifier. The influence of the pH on the retention times of efavirenz and nevirapine was established. With an increase of the pH of the eluent, the capacity factor of efavirenz decreased. Furthermore, at constant pH, the increase of molarity of triethylamine reduced the retention time of efavirenz. A mobile phase consisting of 25 m*M* triethylamine in water–acetonitrile (65:35, v/v) with a pH of 11.7 yielded the best separation of efavirenz and nevirapine from endogenous plasma compounds with short retention times and was therefore chosen.

The UV spectra of efavirenz and nevirapine in 30% acetonitrile and in eluent were recorded. For nevirapine the maximal absorbance was measured at 282 nm. With efavirenz a pH-shift was seen. In 30% acetonitrile and in eluent the maximal absorbance was measured at 248 nm and 269 nm, respectively. Therefore, UV detection at 275 nm was chosen for the detection of both efavirenz and nevirapine.

An internal standard was used to improve robustness. Carbamazepine was selected as internal standard because the drug is structurally allied to nevirapine. Besides, carbamazepine showed proper elution characteristics and was detectable at the same wavelength as efavirenz and nevirapine.

Retention times of nevirapine, carbamazepine and efavirenz were 2.8, 5.0 and 7.8 min, respectively. Total run time was 10 min. Representative selected chromatograms of control human plasma and spiked quality controls with internal standard are shown in Fig. 2.

3.2. Response functions

Details of the calibration curves are shown in Table 1. The lowest total bias and most constant bias across the concentration range were obtained using a weighting factor of $1/x^2$. For each calibration curve the calibration concentrations were back calculated from the response. The deviation for all concentrations from the nominal concentration for all concentrations was between −3.92 and 2.38% for efavirenz and between −5.89 and 4.16% for nevirapine. The C.V. values ranged from 0.75 to 7.45% for efavirenz and from 0.97 to 6.51% for nevirapine.

3.3. Accuracy and precision

The assay performance data for the determination of efavirenz and nevirapine in plasma are presented in Table 2. The intra- and inter-assay accuracies for all tested efavirenz concentrations were between −14.2 and −10.4% for the LLQ and between −5.0 and 8.0% for all other concentrations. Precisions were less than 8.0% for all tested concentrations. The intra- and inter-assay accuracies for all tested nevirapine concentrations were between 6.5 and 12.5% for the LLQ and between −1.4 and 6.5% for all other concentrations. Precisions were less than 6.7% for all tested concentrations.

3.4. Specificity and selectivity

Blank plasma from six different individuals showed no interfering endogenous compounds. Potentially co-administered drugs or metabolites tested had retention times that were different from the

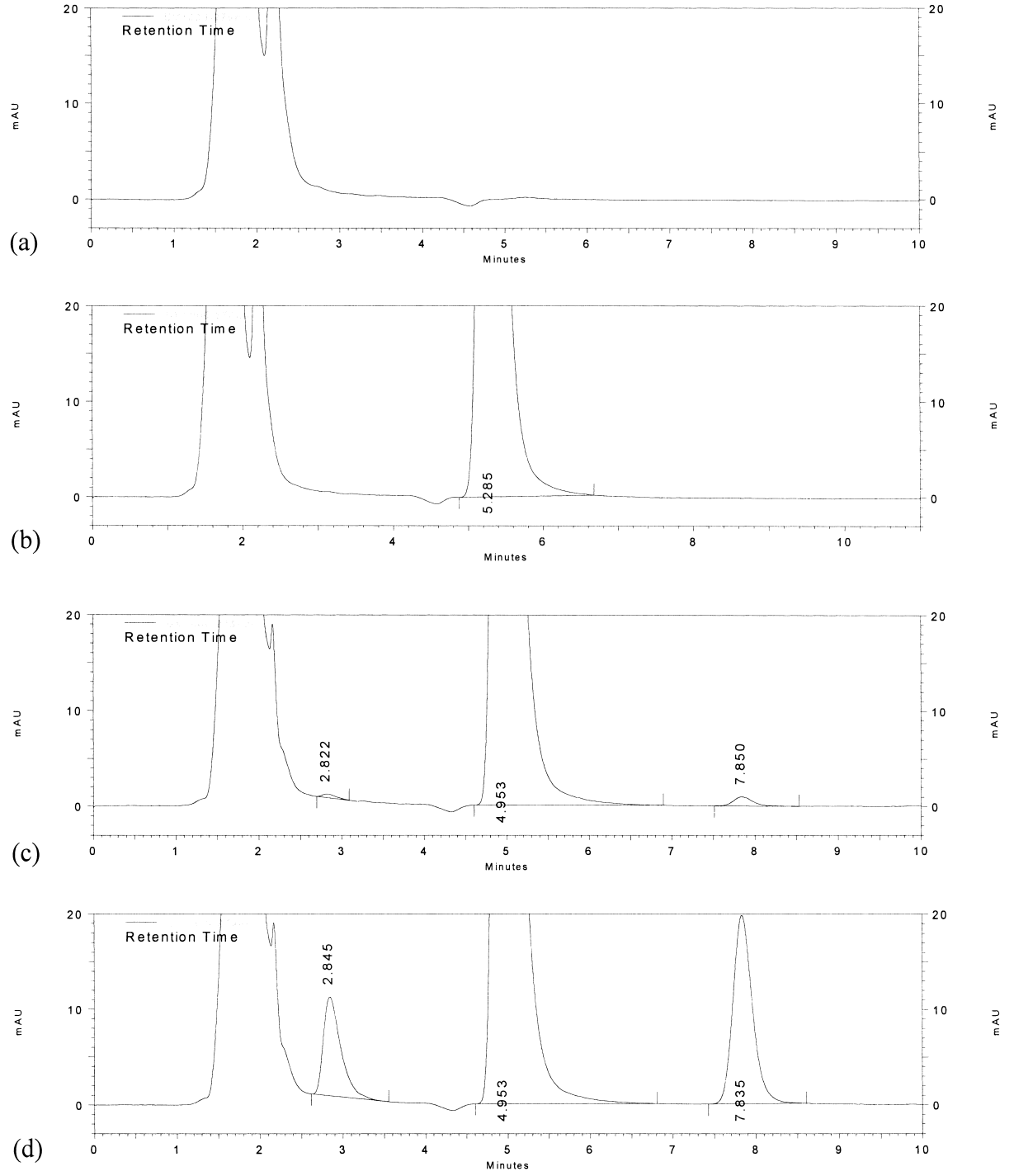


Fig. 2. Selected chromatograms of efavirenz (retention time 7.8 min) and nevirapine (retention time 2.8 min) from control human plasma (a), blank (b) and two quality controls (0.25 and 5.0 mg l^{-1}) with internal standard (retention time 5.0 min) (c, d).

Table 1
Calibration concentrations back calculated from the responses

Run	0.0517	0.1034	0.2585	0.5169	1.034	2.585	5.169	7.754	10.34	15.51
Efavirenz concentration (mg l^{-1})										
1	0.0476	0.1065	0.2515	0.4668	1.011	2.605	5.147	7.852	10.39	15.86
	0.0572	0.0987	0.2495	0.5143	1.074	2.619	5.196	7.932	10.58	15.89
2	0.0561	0.1025	0.2496	0.5193	1.030	2.667	5.199	7.967	10.40	15.66
	0.0487	0.1050	0.2352	0.4805	1.025	2.575	5.247	8.006	10.60	15.77
3	0.0527	0.1052	0.2544	0.4806	1.005	2.580	5.173	7.885	10.42	15.44
	0.0509	0.1022	0.2603	0.5184	1.056	2.628	5.119	7.986	10.27	15.83
Mean	0.0522	0.1034	0.2501	0.4967	1.034	2.612	5.180	7.938	10.44	15.74
SD	0.00	0.00	0.01	0.02	0.03	0.03	0.04	0.06	0.13	0.17
DEV. (%)	0.99	−0.03	−3.24	−3.92	−0.02	1.08	0.22	2.38	1.01	1.51
C.V. (%)	7.45	2.73	3.33	4.69	2.56	1.30	0.86	0.75	1.20	1.07

Run	0.2528	0.5056	1.011	2.528	5.056	7.584	10.11	15.17
Nevirapine concentration (mg l^{-1})								
1	0.2723	0.4685	0.9752	2.461	5.006	7.689	10.28	15.77
	0.2505	0.4887	0.9835	2.563	5.057	7.651	10.29	15.59
2	0.2603	0.4897	0.9507	2.462	5.062	7.763	10.53	15.59
	0.2690	0.4547	0.9594	2.456	5.120	7.826	10.45	15.65
3	0.2396	0.4661	0.9219	2.444	5.002	7.767	10.35	15.60
	0.2882	0.4872	0.9739	2.523	4.973	7.955	10.43	16.03
Mean	0.2633	0.4758	0.9608	2.485	5.037	7.775	10.39	15.71
SD	0.02	0.01	0.02	0.05	0.05	0.11	0.10	0.17
DEV. (%)	4.16	−5.89	−4.98	−1.71	−0.38	2.52	2.73	3.54
C.V. (%)	6.51	3.09	2.33	1.90	1.06	1.39	0.97	1.11

SD: Standard Deviation.
DEV.: Deviation.
C.V.: Coefficient of Variation.

analytes or were not detected with the described analytical method.

3.5. Recovery

The total recoveries for efavirenz were 85.0, 96.6 and 95.8% for the low, mid and high concentration ranges, respectively. The C.V. values ranged from 0.8 to 4.1%. For nevirapine the total recoveries were 86.2, 92.2 and 93.9% for the three concentrations, respectively. The C.V. values ranged from 0.6 to 2.7%.

3.6. Stability

Data on stability of samples and stock solutions are presented in Table 3. Under all conditions tested efavirenz and nevirapine were stable. Deviations were between −7.2 and 12.9% in plasma, between −1.8 and 3.6% in the final extract and between 0.5 and 3.8% in stock solutions.

3.7. Analysis of patient data

The applicability of the assay for pharmacokinetic research in HIV-1-infected individuals was demonstrated by measuring 73 patient samples of efavirenz (600 mg *qd*) or nevirapine (200 mg *bid*). In this population we found plasma concentrations between 0.24 and 5.8 mg l^{-1} for efavirenz and between 1.97 and 11.87 mg l^{-1} for nevirapine. Concentration versus time data of these samples are shown in Fig. 3.

Table 2
Assay performance data

Run	Replicate	val llq efz	qc low efz	qc mid	qc high	>ULQ
		0.1020	0.2551	5.102	12.76	24.90
Efavirenz concentration (mg l^{-1})						
1	1	0.0912	0.2333	5.068	12.93	26.92
	2	0.0952	0.2728	5.163	13.12	27.31
	3	0.0839	0.2486	5.127	13.30	26.17
	4	0.0930	0.2571	5.223	13.19	27.37
	5	0.0782	0.2637	5.304	13.39	26.68
2	1	0.0935	0.2567	5.095	12.98	26.59
	2	0.0890	0.2605	5.244	13.15	26.68
	3	0.0900	0.2419	5.251	12.81	26.68
	4	0.0910	0.2524	5.178	13.04	26.28
	5	0.0936	0.2408	5.328	13.06	27.47
3	1	0.0855	0.2389	5.317	12.84	26.74
	2	0.0855	0.2378	5.217	12.97	26.92
	3	0.0971	0.2412	5.306	12.77	25.92
	4	0.0821	0.2471	5.295	12.86	26.02
	5	0.0873	0.2472	5.262	12.80	26.62
Mean		0.0891	0.2493	5.225	13.01	26.69
Intra-assay accuracy 1 (%)		−13.5	0.0	1.5	3.4	8.0
Intra-assay accuracy 2 (%)		−10.4	−1.8	2.3	2.0	7.4
Intra-assay accuracy 3 (%)		−14.2	−5.0	3.5	0.7	6.2
Inter-assay accuracy (%)		−12.7	−2.3	2.4	2.0	7.2
Mean intra-assay precision		**5.6**	**3.8**	**1.4**	**1.0**	**1.7**
Intra-assay precision 1 (%)		8.0	5.9	1.8	1.4	1.8
Intra-assay precision 2 (%)		2.3	3.5	1.7	1.0	1.6
Intra-assay precision 3 (%)		6.5	1.8	0.8	0.6	1.7
Inter-assay precision (%)		5.9	4.4	1.6	1.4	1.7
		val llq nvp	qc low nvp	qc mid	qc high	>ULQ
		0.2490	0.9960	4.980	12.45	25.51
Nevirapine concentration (mg l^{-1})						
1	1	0.2382	0.9841	5.114	12.83	26.19
	2	0.2812	0.9795	5.157	12.74	25.54
	3	0.2700	0.9782	4.850	12.66	25.16
	4	0.2574	0.9898	5.084	13.31	26.37
	5	0.2797	1.0070	4.839	13.22	26.00
2	1	0.2819	0.9739	5.181	12.86	25.98
	2	0.2916	0.9947	5.270	13.09	26.04
	3	0.2808	0.9749	5.280	12.78	26.11
	4	0.2751	0.9686	5.123	13.16	25.99
	5	0.2709	0.9991	5.357	13.03	26.78
3	1	0.2742	1.0164	5.329	13.13	26.09
	2	0.2531	1.0250	5.252	13.23	26.63
	3	0.2735	1.0349	5.344	12.86	25.30
	4	0.2564	1.0231	5.339	13.26	25.83
	5	0.2693	1.0505	5.265	13.14	26.40
Mean		0.2702	1.0000	5.186	13.02	26.03
Intra-assay accuracy 1 (%)		6.5	−0.8	0.6	4.0	1.3
Intra-assay accuracy 2 (%)		12.5	−1.4	5.3	4.3	2.0
Intra-assay accuracy 3 (%)		6.5	3.4	6.5	5.4	2.4
Inter-assay accuracy (%)		8.5	0.4	4.1	4.6	2.0
Mean intra-assay precision		**4.4**	**1.3**	**1.9**	**1.6**	**1.3**
Intra-assay precision 1 (%)		6.7	1.2	3.0	2.3	1.9
Intra-assay precision 2 (%)		2.8	1.4	1.7	1.2	0.2
Intra-assay precision 3 (%)		3.7	1.3	0.8	1.2	1.7
Inter-assay precision (%)		5.1	2.5	3.2	1.6	1.7

Table 3
(a) Stability of efavirenz and (b) stability of nevirapine

Storage condition	conc. (mg l^{-1})	recovery (%)	RSD (%)	*n*
(a) Stability data of efavirenz				
Plasma				
24 h at ambient temperatures	0.2551	99.5	0.3	3
	12.76	100.4	−1.3	3
7 days at 4 °C	0.2551	87.1	−7.2	3
	12.76	99.8	−1.0	3
Three freeze–thaw cycles	0.2551	97.5	1.7	3
	12.76	100.0	−0.4	3
6 months at −20 °C	0.2468	107.1	12.9	2
	0.9870	103.2	2.3	2
	3.948	98.8	5.3	2
	7.896	95.0	−2.2	2
Final extract				
5 days at ambient temperatures	0.2551	92.1	−1.8	3
	12.76	99.9	−1.0	3
Stock solutions				
24 h at ambient temperatures	996.00	103.8	3.8	3
24 months at −20 °C	996.00	102.3	2.3	3
(b) Stability data of nevirapine				
Plasma				
24 h at ambient temperatures	0.9960	99.7	1.2	3
	14.45	89.4	0.1	3
7 days at 4 °C	0.9960	99.9	−3.0	3
	14.45	90.8	0.4	3
Three freeze–thaw cycles	0.9960	105.8	1.3	3
	14.45	92.1	0.2	3
12 months at −20 °C	0.5056	104.6	4.6	2
	1.011	106.6	6.6	2
	2.528	102.3	2.3	2
	5.056	93.3	−6.7	2
	7.584	97.2	−2.7	2
	10.11	100.9	0.9	2
	15.17	96.8	−3.2	2
Final extract				
5 days at ambient temperatures	0.9960	106.6	3.6	3
	14.45	91.3	1.0	3
Stock solutions				
24 h at ambient temperatures	1020.4	100.5	0.5	3
36 months at −20 °C	1020.4	104.3	4.3	3

4. Discussion and conclusions

In conclusion, a simple and rapid assay was developed and validated, according to FDA guidelines, for the simultaneous determination of efavirenz and nevirapine in human plasma. 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. A small aliquot of plasma and simple and easily available instrumentation are used. The assay proved to be accurate and precise and is currently used for therapeutic drug monitoring and pharmacokinetic research in our institute.

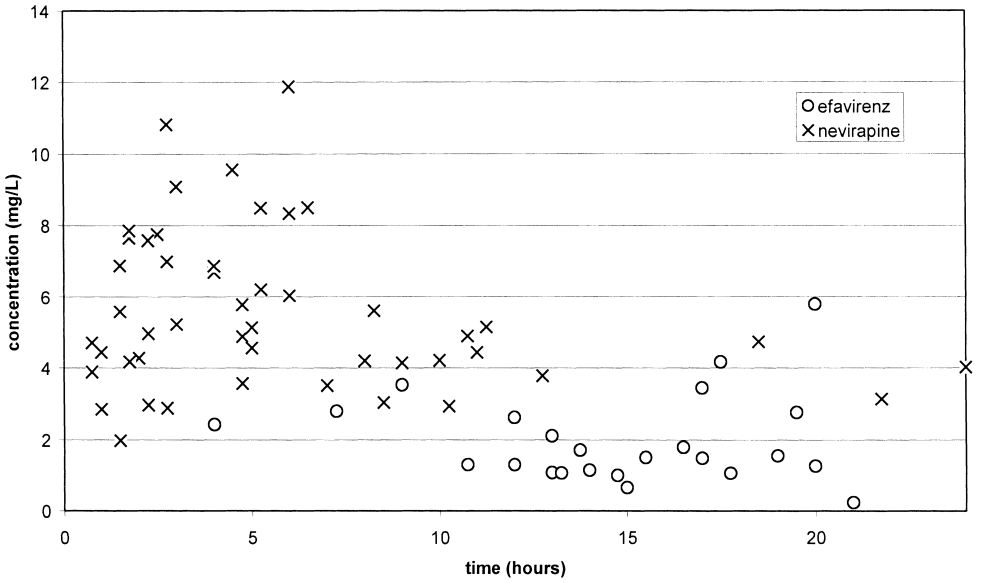


Fig. 3. Concentration versus time data of efavirenz and nevirapine in plasma samples from HIV-1-infected patients.

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