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Rapid determination of nevirapine in human plasma by ion-pair reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

Nevirapine is a non-nucleoside reverse transcriptase inhibitor for the treatment of HIV-1-infected patients. A simple and rapid high-performance liquid chromatographic method for the quantification of nevirapine in human plasma is described. Sample pretreatment consists of protein precipitation with acetonitrile. The analyte is separated from endogenous compounds by isocratic reversed-phase, ion-pair, high-performance liquid chromatography with ultraviolet detection at 282 nm. The method has been validated over the range of 52–10 400 ng/ml using 250 μl of plasma. The assay was linear over this concentration range. Within- and between-day precisions were less than 4.5% for all quality control samples. The lower limit of quantitation was 52 ng/ml. Recovery of nevirapine from human plasma was 94.5%. This validated assay is suited for use in pharmacokinetic studies with nevirapine and can readily be used in a hospital laboratory for the monitoring of nevirapine concentrations.

Keywords: Nevirapine

1. Introduction

Nevirapine (BI-RG-587, Viramune®, Fig. 1) belongs to the class of non-nucleoside reverse transcriptase inhibitors. It is the first member of its class to be approved in the USA for use in patients with HIV-1 infection. Nevirapine is a noncompetitive inhibitor of the viral replicative enzyme reverse transcriptase, an important therapeutic target for the treatment of HIV-1 infection [1]. Nevirapine does not inhibit HIV-2 reverse transcriptase [1].

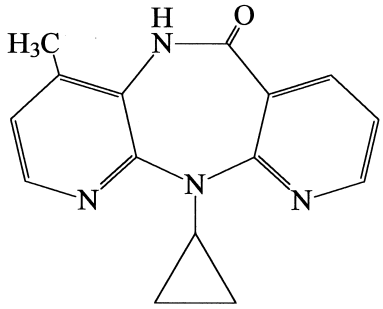

Fig. 1. Molecular structure of nevirapine.

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Treatment of patients with nevirapine monotherapy rapidly selects for resistant isolates of HIV-1, most of which contain a tyrosine-to-cysteine mutation at residue 181 (Y181C) of the reverse transcriptase enzyme [2]. However, when given in combination with one or more nucleoside reverse transcriptase inhibitors nevirapine has demonstrated a potent antiviral effect [1].

The pharmacokinetic properties of nevirapine in HIV-1 infected patients are largely unknown. Nevirapine has been shown to induce its own cytochrome P450 mediated metabolism, resulting in a decrease in the terminal phase half-life to 25–30 h following multiple dosing with 200–400 mg/day [1,3]. The currently recommended dose for nevirapine is 200 mg once daily during a lead-in period of 2 weeks, followed by 200 mg twice daily.

We report the development and validation of an ion-pair high-performance liquid chromatographic (HPLC) assay with ultraviolet detection for the quantitative determination of nevirapine in human plasma. To our knowledge, no detailed methodology for the quantitative determination of nevirapine in plasma has been described thus far. The presented assay can be used to obtain pharmacokinetic data in HIV-1 infected patients.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Model 8800 solvent delivery pump (Spectra Physics, San Jose, CA, USA), a Model 8880 automatic sample injection device, a Spectra 100 variable wavelength detector, and a Chromjet® integrator (all Spectra Physics). The analytical column was a C_8-column (150×4.6 mm I.D., 5 μm particle size; Jones Chromatography, Lakewood, CO, USA) protected by a Chromguard® C_{18} guardcolumn (10×3 mm I.D., Chrompack, Middelburg, The Netherlands). Analytical runs were processed by the PC 1000 SYSTEM software (version 3.01, Spectra Physics). UV-spectra of nevirapine in methanol were recorded with a Model 918 UV–VIS spectrophotometer (GBC Scientific Equipment, Dandenons, Australia).

2.2. Chemicals

Nevirapine was kindly provided by Boehringer Ingelheim (Lot K, Ridgefield, CT, USA). Acetonitrile and methanol (both HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Dimethylsulfoxide, potassium dihydrogenphosphate, sodium hydroxide and hexane-1-sulfonic acid (sodium salt) analytical reagent grade, were 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 nevirapine were prepared by dissolving the appropriate amount of the compound, accurately weighed, in dimethylsulfoxide to yield a concentration of 1.0 mg/ml. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital), 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 nevirapine was diluted with methanol. For the construction of each calibration curve nine spiked plasma samples were analyzed in duplicate. Calibration concentrations of 52–10 400 ng/ml nevirapine in plasma were prepared in eppendorf tubes by adding diluted stock solutions to human plasma. The solutions were 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 30 s and centrifuged for 3 min at 10 500 *g*. The clear supernatants were transferred to autosampler vials with inserts.

A second stock solution with separate weighing was used for preparation of quality control samples

(QCs) yielding concentrations of 53, 5300 and 10 600 ng/ml nevirapine in plasma. The QCs were processed further identically to the calibration samples.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature on a C_8 analytical column with a mobile phase composed of 25 m*M* phosphate buffer (pH 5.5)–methanol–acetonitrile (7:2:1, v/v/v) containing 25 m*M* hexane-1-sulfonic acid (final concentration in mobile phase). Prior to use, air was removed by passing helium through the mobile phase. Absorbance was measured at 282 nm. The flow-rate was maintained at 1.0 ml/min. Aliquots of 50 μl were injected.

2.6. Specificity and selectivity

The interference of endogenous compounds was investigated by the analysis of six different blank plasma samples. The following compounds, frequently used by HIV-infected patients, were investigated for interference with the analytical method (including sample pretreatment): cotrimoxazole, delavirdine, didanosine, fluconazole, folinic acid, ganciclovir, indinavir, lamivudine, methadone, nelfinavir, 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 and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) was arbitrarily set at 10 400 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precisions of the method were determined by assaying six replicates of each of the QCs in three separate analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within-day 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 $(1/\text{conc.}^2)$ [4,5].

The average recovery of nevirapine over the concentration range of the standard curve was determined in two analytical runs by calculating the ratio of the slopes of a calibration curve in plasma and nonprocessed solutions.

2.9. Stability

Blank plasma was spiked with aliquots of diluted nevirapine stock solution to yield concentrations of 265 and 5300 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 plus three freeze–thaw cycles. For each concentration and each storage condition four replicates were analyzed in one analytical run. The concentration of nevirapine after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

2.10. 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.

3. Results and discussion

3.1. Chromatography and detection

Starting-point for the development of our method was a brief description of an HPLC–UV method for the quantitative determination of nevirapine in plasma by Jayaraj et al. [6].

Reversed-phase chromatography was initially performed with a C_8 analytical column and a mobile phase consisting of 25 m*M* phosphate buffer (pH

6)–methanol–acetonitrile (7:2:1, v/v/v) and hexane-1-sulfonic acid (5 m*M* in mobile phase). Peak shape and separation from endogenous compounds could be optimized by increasing the concentration of hexane-1-sulfonic acid to 25 m*M* and pH adjustment to 5.5. A methanol content of 20% v/v in the mobile phase turned out to be optimal for separation of nevirapine from endogenous plasma compounds. A typical chromatogram of a blank plasma sample and a sample of 242 ng/ml nevirapine in plasma is shown in Fig. 2. The run time of this assay was only 12 min.

The UV spectrum of nevirapine in methanol was recorded. Maximal absorbance at 282 nm was measured (specific extinction 293). Nevirapine demonstrated no significant fluorescence properties in methanol after excitation at 282 nm.

There was no suitable internal standard available at the time of development of the assay. However, the assay gives satisfactory validation results without the use of an internal standard.

3.2. Sample pretreatment and recovery

Jajaray et al. reported solid-phase extraction of aliquots of 250 μl of plasma using Certify I silica cartridges [6]. In our method sample pretreatment is greatly simplified by protein precipitation with acetonitrile and subsequent evaporation of the organic phase to concentrate the analyte.

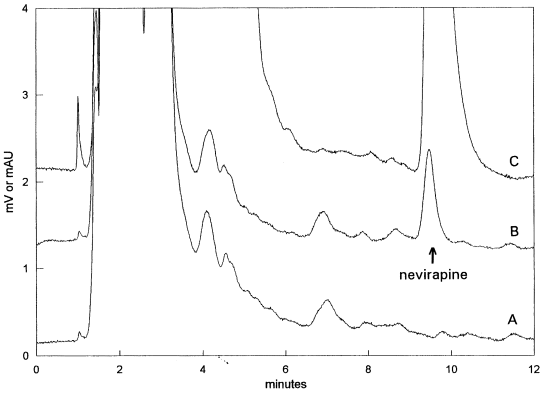

Fig. 2. Chromatograms typical for the analysis of nevirapine: (A) blank plasma, (B) spiked plasma sample of 242 ng/ml and (C) a patient sample of 5240 ng/ml.

Recovery of nevirapine from spiked plasma samples by using this methodology was 94.5±4.7%.

3.3. Specificity and selectivity

Blank plasma from six different individuals showed no interfering endogenous substances eluting at the retention time (t_R) of nevirapine. Potentially coadministered drugs or metabolites tested had retention times that were different from nevirapine (t_R=9.5 min) (cotrimoxazole, t_R=8.8 and 17.5 min, ranitidine, t_R=11.5 min, fluconazole, t_R=13.7 min, folinic acid, t_R=32 min) or were not detected with the described analytical method.

3.4. Limit of quantification

At a concentration of 52 ng/ml the relative standard deviation (R.S.D.) and percent deviation from the nominal concentration were both less than 20%. Thus, 52 ng/ml was defined as the LLQ. At all other concentrations up to the ULQ (10 400 ng/ml) the R.S.D. and percent deviation from the nominal concentration were always less than 15%.

In clinical practice the average steady-state peak and trough concentrations are approximately 7 and 4 μg/ml, respectively [7]. Hence, the concentration range which is required for application of this HPLC methodology in clinical pharmacokinetic studies of nevirapine is covered.

3.5. Validation: accuracy, precision, linearity and stability

Accuracies and precisions for the quantitation of nevirapine in human plasma are listed in Table 1. Using the peak area in combination with a weight factor ($1/\text{conc.}^2$) resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at three concentrations 97.3–105.2% of the nominal concentrations) and precise (within-day precision ranged from 1.9–4.5% and between-day precision ranged from 0.8 to 3.0%). Correlation coefficients (r^2) of calibration curves were >0.995 as determined by least-squares analysis.

Calibration curves proved to be linear in the range 52–10 400 ng/ml with the use of the *F*-test for lack

Table 1
Accuracy and precision for the analysis of nevirapine in spiked human plasma samples

Concentration (ng/ml)	Accuracy (%)	Precision		n[a]
		Between-day	Within-day	
265	97.3	0.8	4.5	18
5300	105.2	3.0	2.2	18
10 600	100.6	2.1	1.9	18

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

of fit as an indicator of linearity of the regression model ($P<0.05$).

The stability of nevirapine at various conditions is shown in Table 2. Under all conditions tested nevirapine is stable with concentrations of at least 94.8% of the initial concentration.

4. Conclusion

In conclusion, a rapid, sensitive, specific and validated assay for the quantitative determination of nevirapine in human plasma is described. The applicability of the assay for pharmacokinetic research in HIV-1 infected patients is demonstrated by analysis of plasma samples from 11 HIV-1 infected patients at steady-state, 4 h after ingestion of 200 mg nevirapine. The average plasma nevirapine concentration of 5.01±2.12 mg/l was in agreement with concentrations reported by Havlir et al. [7]. The assay meets all current requirements for the validation of a bioanalytical methodology. This HPLC assay can be used for pharmacokinetic studies with nevirapine in HIV-infected patients and can readily be used in any hospital laboratory for the monitoring of nevirapine plasma concentrations.

Table 2
Stability of nevirapine in spiked human plasma samples

Storage conditions	Conc. (ng/ml)	Recovery (%)	C.V.[a] (%)	n[b]
1 h at 60°C	265	115.4	6.7	4
	5300	100.9	2.1	4
24 h at 24°C	265	96.1	9.0	4
	5300	98.0	2.0	4
7 days at 4°C	265	103.6	3.1	4
	5300	96.9	1.2	4
30 days at −30°C	265	106.4	4.1	4
	5300	94.8	2.6	4
Three freeze–thaw cycles	265	106.6	6.0	4
	5300	96.2	1.2	4

[a] C.V.=coefficient of variation.
[b] n=Number of replicates.

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