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Short communication

Simple and rapid determination of nevirapine in human serum by reversed-phase high-performance liquid chromatography

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Abstract

Nevirapine is an antiretroviral agent belonging to the class of non-nucleoside reverse transcriptase inhibitors. We describe a fast, simple isocratic reversed-phase high-performance liquid chromatography method with a 30-mm long column for assaying nevirapine in human serum. After deproteinization of 200 μl serum samples with 50% trichloroacetic acid, the supernatant was injected into a reversed-phase C_{18} column, using 10 m*M* phosphate buffer (pH 5)–acetonitrile (82:18, v/v) as the mobile phase. Peak detection was performed at 240 nm. Nevirapine retention time was 2 min. The method was validated over 0.1–10 μg/ml and the assay was linear over this concentration range (r^2>0.998). Within- and between-day precisions were less than 5.4%. The lower limit of quantification was 0.1 μg/ml. Nevirapine in human serum samples was stable for 2 days at 20–25°C, 15 days at 4°C and 3 months at −20°C.

Keywords: Nevirapine

1. Introduction

Nevirapine (Viramune) is the first member of a new therapeutic class of drugs (NNRTIs: non-nucleoside reverse transcriptase inhibitors) for the treatment of human immunodeficiency virus (HIV) infection [1]. Treatment with nevirapine monotherapy is notorious for rapidly eliciting resistance due to mutations of the amino acids surrounding the NNRTI binding site [2]. However, in association with two other antiretroviral products, nucleoside reverse transcriptase inhibitors (NRTIs) and/or protease inhibitors (PIs), nevirapine significantly reduces the viral load and increases CD4 cell count, particularly in treatment-naive patients [1].

Nevirapine is metabolized by cytochrome P450 (CYP3A4) and is a relatively potent inductor of the enzyme; consequently, it has the ability to reduce plasma concentrations of other drugs that are also biotransformed by CYP3A4 as PIs [3]. Therapeutic drug monitoring of nevirapine may be warranted to prevent or delay the occurrence of viral resistance, and to ensure optimal therapy for HIV-infected patients [4].

Few methods have been described for nevirapine determination by high-performance liquid chromatography (HPLC) [5–8]. The chromatographic methods reported in the literature use conventional col-

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umns (150 mm length) and retention times of 5 to 12 min.

This report describes the development and validation of an ion-pair isocratic reversed-phase (HPLC) assay, using a 30-mm long column and ultraviolet detection, for the quantitative determination of nevirapine in human serum. The procedure was evaluated in a clinical setting to determine its usefulness for monitoring serum levels in patients receiving nevirapine treatment.

2. Experimental

2.1. Chemicals and reagents

Nevirapine was kindly provided by Boehringer Ingelheim (lot RM-1439; Ridgefield, CT, USA). Acetonitrile, methanol, triethylamine, sodium dihydrogenphosphate monohydrate, orthophosphoric acid (85%) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Blank, drug-free serum was obtained from patients not receiving antiretroviral drugs. All chromatographic solvents were of HPLC grade, and all other chemicals were of analytical grade. Water was purified by use of the Milli-Q Labo System (Millipore, Molsheim, France).

2.2. Instrumentation and chromatographic conditions

The HPLC system (Kontron Instruments, Milan, Italy) was equipped with a Model 325 solvent-delivery system, a Model 465 automated sample injector with variable-injection volume, and a Model 432 ultraviolet absorption variable-wavelength detector with an 8-μl flow cell. We used a Spherisorb C_{18} analytical column (30×4.6 mm I.D., 3 μm particle size; Perkin-Elmer, Norwalk, CT, USA). Data acquisition, integration and data calculation were performed with an Acer 1120 SX computer with Kontron PC-integrator software, version 3.00. UV spectra of nevirapine in the mobile phase were recorded with a Model 860 UV–Vis spectrophotometer (Kontron Instruments). The chromatographic analysis was performed at room temperature with isocratic elution. The mobile phase consisted of 10 m*M* phosphate buffer (containing 10 m*M* triethylamine, pH 5)–acetonitrile (82:18, v/v), which was degassed by filtering through a 0.45-μm (pore size) membrane filter (Millipore, Milford, MA, USA) before use. The pump was run at a flow-rate of 1.0 ml/min. A sample volume of 50 μl was injected in duplicate into the column. The UV detector was operated at a wavelength of 240 nm and 0.02 absorbance units full scale (AUFS).

2.3. Standard preparation

Nevirapine stock solution (0.5 mg/ml) was prepared by dissolving 10 mg of the drug in 10 ml of methanol solution. Working standards to study recovery were obtained by diluting the stock solution with methanol–water (50:50, v/v) to concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/ml. To prepare the calibration samples, the stock solution of nevirapine was diluted with methanol–water (50:50, v/v). Calibration concentrations of 0.1–10 μg/ml nevirapine in serum were prepared in Eppendorf tubes by adding 100 μl diluted stock solution to 900 μl of drug-free human serum. All the solutions and calibration standards were stored at −80°C until analysis.

2.4. Sample pretreatment

The samples were deproteinized by adding 20 μl of 50% TCA to 200 μl of serum, vortex-mixing for 30 s and centrifuging at 10 500 *g* for 5 min. The supernatant was injected in duplicate into the column. The calibration standards and quality control samples were assayed in the same manner.

2.5. Analytical recovery and precision

Recovery was determined by comparing of the peak area of nevirapine from deproteinized calibration standards (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/ml) with the respective non-extracted standard solutions [nevirapine in methanol–water (50:50, v/v)] at the same concentrations in five separate runs.

Within-day variation was determined by assaying aliquots of a blank serum sample spiked with

nevirapine at concentrations of 0.4, 2 and 7.5 μg/ml, 10 times in the same run. Day-to-day variation was calculated by assaying the same samples once a day for 10 days.

2.6. Stability

Sample stability at room temperature (20–25°C), 4°C and −20°C was determined by assaying quality control serum samples spiked with 0.4 and 2 μg/ml of nevirapine. Twenty-two aliquots were prepared for each concentration. One, immediately stored at −80°C at time zero, was considered the 100% reference value. One aliquot of each concentration maintained at each temperature condition was withdrawn at 1, 2, 7, 15 days and 1, 2 and 3 months and stored at −80°C until analysis. The percentage changes in nevirapine concentration over the study were compared with the actual concentration at time zero. A decrease from the initial concentration higher than 10% was considered to represent a significant loss of drug.

2.7. Selectivity

We investigated potential interferences with drugs frequently combined with nevirapine in HIV-positive patients, such as NRTIs, PIs and others. The following drugs for the interference analyses were obtained from the manufacturers: didanosine (Bristol Mayers), indinavir (Merck Sharp & Dohme), ritonavir (Abbott Laboratories), saquinavir and nelfinavir (Roche), sulfametoxazol and trimetoprim (Wellcome). Zidovudine, zalcitabine, estavudine, lamivudine and fluconazol were obtained from the Hospital Pharmacy (Vall d'Hebron General Hospital, Barcelona, Spain). The solid material was dissolved in methanol and a final concentration of 20 μg/ml was obtained by dilution with methanol–water (50:50, v/v).

2.8. Application

The clinical applicability of our HPLC method was investigated by analyzing nevirapine in serum samples from HIV-positive patients treated with nevirapine at a dosage regimen of 200 mg orally twice daily. Blood samples ($n=10$) were drawn at 30 min before dose in steady state conditions. Serum samples were stored at −80°C until assayed.

2.9. Calibration and statistical analysis

Calibration curves were obtained by plotting the peak area versus the nominal nevirapine concentrations. The values from the linear regression were used for calculating nevirapine concentrations in the samples from their peak area.

The linear regression equation without weight between the peak-area and the concentration of calibration curves were performed with the SPSS for Windows, version 8.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Development method

The starting point for the development of our method was a brief description of a HPLC–UV method for quantitative determination of nevirapine in plasma by van Heewijk et al. [8]. The reversed-phase chromatography was initially performed with a mobile phase consisting of 10 m*M* phosphate buffer (pH 5.5)–methanol–acetonitrile (70:20:10, v/v/v) and triethylamine (10 m*M* in phosphate buffer). Peak shape and separation from endogenous compounds were optimized without methanol in the mobile phase and pH was adjusted to 5. Assays performed on drug-free serum samples showed the absence of any interfering endogenous peaks. The UV spectrum of nevirapine in the mobile phase demonstrated maximum absorbance at 240 and 282 nm. We selected 240 nm because higher peak chromatographic areas were achieved at this wavelength. There is a suitable internal standard available for nevirapine determination; however, the assay gave satisfactory validation results without the use of an internal standard.

Fig. 1 shows representative chromatograms of a blank serum sample and a serum sample from a patient receiving nevirapine obtained with this pro-

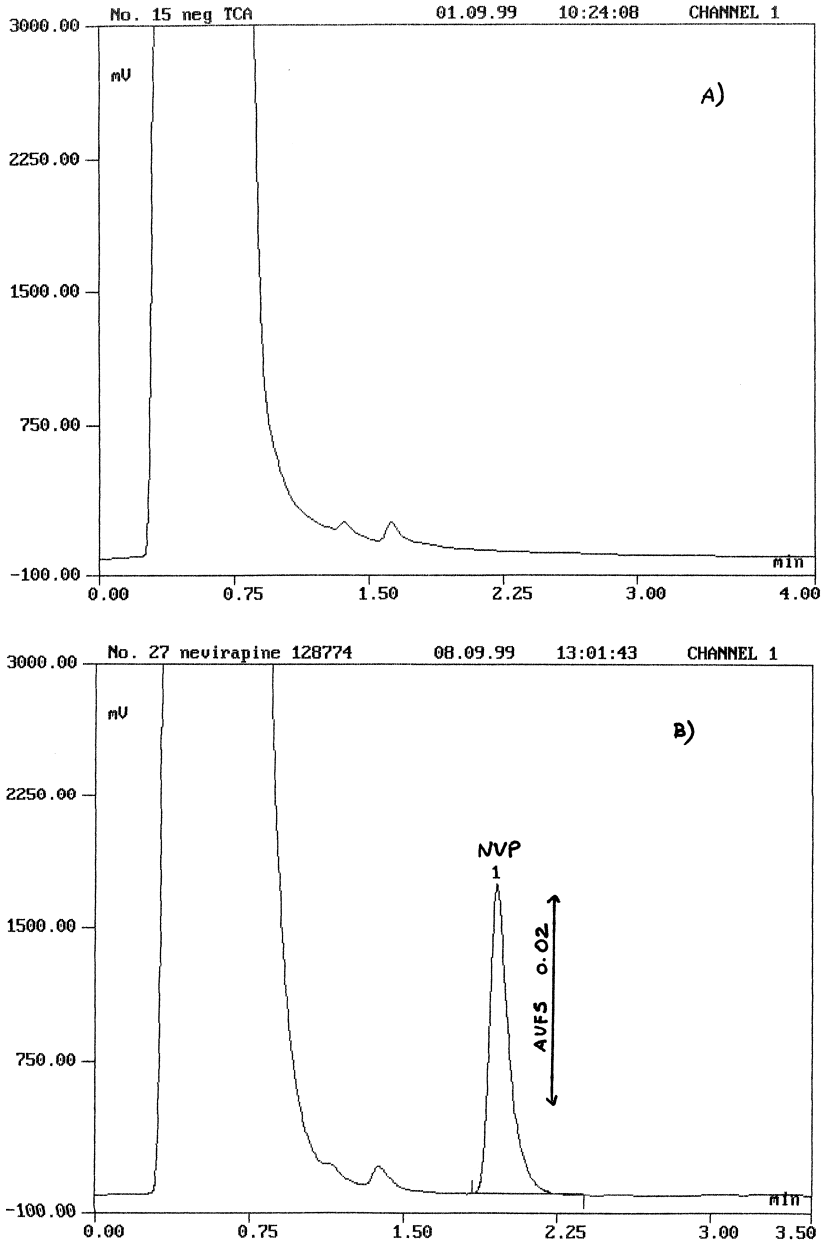

Fig. 1. Chromatograms of (A) a blank serum sample and (B) a serum sample from a patient containing 3.98 μg/ml nevirapine (t_R, 2 min).

cedure. The retention time for nevirapine was 2 min and the assay run time was only 3 min.

3.2. Sample pretreatment

Several extraction procedures have been described previously: solid-phase extraction with silica cartridges [5], liquid–liquid extraction with dichloromethane and chloroform–isopropanol [6,7] and protein precipitation with acetonitrile and subsequent evaporation of the organic phase to concentrate the analyte [8]. With our method, sample pretreatment is greatly simplified by protein precipitation with 50% TCA and direct injection of the supernatant.

Table 1
Precision of the HPLC assay of nevirapine ($n=10$)

Concentration (μg/ml)			RSD (%)
Added	Found ($x\pm$SD)	Range	
Within-day			
0.4	0.35±0.019	0.31–0.36	5.4
2	1.78±0.024	1.74–1.82	1.3
7.5	7.69±0.127	7.49–7.98	1.6
Day-to-day			
0.4	0.36±0.016	0.34–0.39	4.4
2	1.77±0.052	1.66–1.87	2.9
7.5	7.62±0.163	7.37–7.81	2.1

3.3. Recovery and precision

The mean recoveries from the precipitation procedure ranged from 66.1±1.3% at 1 μg/ml to 77.6±2.5% at 2.5 μg/ml. Mean recovery over the range of 0.1 to 10 μg/ml, was 70.6% ($n=35$). The relatively low recovery of nevirapine had no negative effects on assay performance. The within-day and day-to-day relative standard deviations (RSDs) were less than 5.4% and 4.4%, respectively (Table 1).

3.4. Linearity and limit of detection

The linearity was verified from 0.1 μg/ml to 10 μg/ml. The lower limit of detection at a signal-to-noise ratio of 3 was 0.1 μg/ml.

The mean linear regression equation between the peak-area (y) and the concentration (x) was $y=46.1573x-1.1173$ ($n=10$). Correlation coefficients (r^2) of the calibration curves were $r^2>0.998$ as determined by least-squares analysis.

3.5. Stability

The stability of nevirapine under various conditions at two concentrations is shown in Table 2. As may be seen, the nevirapine concentration remained between 90 and 110% of the initial concentration for 2 days at room temperature (20–25°C), 15 days at 4°C and 3 months at −20°C.

3.6. Selectivity and application

Chromatographic analysis of the drugs that are frequently prescribed for HIV-positive patients with this method showed no detection of any drug. The median (range) nevirapine concentration in serum samples at 30 min before dose was 5.56 μg/ml (1.81–11.68).

4. Discussion

All the chromatographic methods previously described for the determination of nevirapine in plasma have used conventional columns [5–8]. The described retention times are 5 to 12 min. The use of short columns (30 mm×4.6 mm I.D.) presents a series of advantages over these techniques – fast equilibrium, less retention time, and lower consumption of the mobile phase – factors that are important in evaluating the practicability of the method. Although the sensitivity of our method is not as high as that reported by others (0.1 μg/ml vs. 0.052 μg/ml) [8], it is sufficient for monitoring serum nevirapine concentrations along the dosage interval.

Table 2
Stability of serum samples containing nevirapine

Temperature (°C)	Nevirapine concentration (μg/ml)		% Initial drug concentrations remaining						
	Theoretical	Actual	1 day	2 days	7 days	15 days	1 month	2 months	3 months
20–25	0.4	0.38	99.1	93.7	87.5	84.1	83.8	83.5	64.8
4	0.4	0.38	98.5	100.6	99.1	92.6	86.7	81.3	77.5
−20	0.4	0.38	100.7	99.5	100.2	97.7	101.5	99.5	98.5
20–25	2	1.87	101.1	95.1	80.8	78.1	76.9	71.7	67.8
4	2	1.87	99.8	100.2	99.8	92.8	89.3	86.5	85.6
−20	2	1.87	97.3	98.8	97.5	100.0	97.1	97.7	96.4

5. Conclusion

In conclusion, a simple, rapid, sensitive and validated assay for the quantitative determination of nevirapine in human serum is described. 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 serum concentrations.

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