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HPLC-UV method for the quantitation of nevirapine in biological matrices following solid phase extraction

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Abstract

Nevirapine (VIRAMUNE®) is a non-nucleoside reverse transcriptase inhibitor with activity against human immunodeficiency virus type 1 (HIV-1), currently marketed for the treatment of HIV-1 infected adults. A reverse phase HPLC-UV method was optimized and validated for the determination of nevirapine in human plasma, serum, milk and cerebrospinal fluid. The analyte was extracted from 250 μl of biofluid using a bonded silica solid phase extraction column, and resolved chromatographically on a reversed-phase, 15 × 0.46 cm i.d. 5 μm particle Supelco LC-8 analytical column with an isocratic mobile phase of 63% phosphate buffer (0.025 M, pH 6.0) with 1-butanesulfonic acid as anion-pair reagent: 21.5% methanol: 15.5% acetonitrile. The peaks were detected at a flow rate of 1.0 ml min^{-1}, at a wavelength of 280 nm, with a run time of 10 min. The assay was linear over a range of 25 to 10 000 ng ml^{-1}. This method has been used for the clinical development of nevirapine.

Keywords: Nevirapine; Reversed-phase chromatography; Human plasma; Human serum; Human milk; Human cerebrospinal fluid

1. Introduction

Nevirapine, 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2′,3′-e][1,4]diazepin-6-one, is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of human immunodeficiency virus type 1 (HIV-1). The structure of nevirapine is shown in Fig. 1. It binds directly to an allosteric site on reverse transcriptase and inhibits both the RNA- and DNA-dependent DNA polymerase activities. The fused ring system produces a UV spectrum with a flat portion of the UV curve at a wavelength of 280 nm. A molar extinction coefficient of 7.7×10^3 was obtained at this wavelength. The high absorption maximum makes nevirapine a suitable candidate for ultraviolet detection after solid phase or liquid–liquid extraction from biological matrices followed by HPLC separation. Authentic standards of nevirapine and BIRH 414, a chemical analogue of nevirapine in which the

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cyclopropyl group is replaced by an ethyl group, can be chromatographically separated on a reversed phase C_8 column.

An HPLC method was presented previously for the support of preclinical toxicology samples and phase I human PK studies [1]. The method presented here describes the validation of an HPLC-UV method for the determination of nevirapine concentrations in human plasma, serum, milk and cerebrospinal fluid following solid phase extraction. This method has been used to support the clinical development of nevirapine for phases I through IV. Portions of this method have been briefly described in abstract form [2]. A separate HPLC method has been validated for the simultaneous determination of nevirapine and its 12-hydroxy metabolite in human urine [3].

2. Materials and methods

2.1. Chemicals

Nevirapine (BIRG-587) and BIRH-414, a chemical analogue of nevirapine in which an ethyl group replaces the cyclopropyl group, were obtained from the Analytical Chemistry Department of Boehringer Ingelheim Pharmaceuticals, Inc. in Ridgefield, CT. The 1-butanesulfonic acid (sodium salt) was purchased from Sigma. Human heparinized blank control plasma came from Biological Speciality Corporation. USP grade saline, 0.9% sodium chloride, was supplied by Baxter. Bond Elut Certify cartridges were from Varian. All other HPLC grade chemicals were obtained from EM Science, including methanol, ethyl acetate, acetonitrile, and water. Acetic acid, potassium phosphate monobasic, potassium phosphate dibasic, sodium hydroxide, o-phosphoric acid, and ammonium hydroxide were Reagent grade from EM Science.

2.2. Preparation of reagents and standard solutions

All solutions were stored at room temperature. Phosphate buffer for extraction was prepared by adjusting potassium phosphate solution (0.1 M) to pH 2.0 with o-phosphoric acid. Ammonium hydroxide (2% v:v) was prepared daily in ethyl acetate with sonication. Mobile phase phosphate buffer (0.025 M) with 1-butanesulfonic acid sodium salt (0.0052 M) was prepared and adjusted to pH 6.0 by the addition of sodium hydroxide (10 N). Duplicate weighings of nevirapine were made with one being used exclusively for calibration curves and the other being used to prepare the quality control samples. Nevirapine (100, 25, and 2.5 μg ml^{-1}) and BIRH-414 (100 and 15 μg ml^{-1}) stock solutions were prepared in methanol and stored at room temperature for 4 months. Plasma and quality control samples were stored at -20°C.

2.3. Chromatography conditions

The analyte was resolved chromatographically on a thermostated (30°C) reversed phase, 15 × 0.46-cm i.d. 5 μM particle Supelco LC-8 analytical column, protected by an LC-8 guard column. An isocratic mobile phase consisted of phosphate buffer (0.025 M, pH 6.0) with (0.025 M) 1-butanesulfonic acid as an ion-pair reagent: methanol: acetonitrile (63:21.5:15.5, v/v/v). The ion-pair reagent was used to separate nevirapine from endogenous plasma components. The peaks were detected at a flow rate of ≈ 1.0 ml min^{-1} by UV absorbance at a wavelength of 280 nm and with a total run time of 10 min.

2.4. Sample preparation for the extraction procedure

Unknown samples, quality control samples, and blank heparinized plasma were allowed to thaw at room temperature. 50 μl of 15 μg ml^{-1}

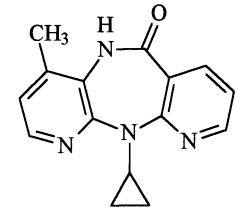

Fig. 1. Structure of nevirapine.

Table 1
Intraday and interday reproducibility of nevirapine quality control standards

Concentration (ng ml^{-1})	Intraday reproducibility			Interday reproducibility		
	n	Mean + SD (ng ml^{-1})	RSD (%)	*n*	Mean + SD (ng ml^{-1})	RSD (%)
100	6	107 ± 4.9	4.6	24	111 ± 12.3	11.1
2000	6	2019 ± 70.7	3.5	25	2010 ± 0.5	4.1
3000	6	2607 ± 72.0	2.8	25	3034 ± 1.1	11.1
7000	6	6625 ± 196.3	3.0	25	6945 ± 0.8	6.0

Table 2
Nevirapine freeze/thaw cycles

Mean quality control standard concentrations						
Freeze/thaw	150 ng ml^{-1}		1500 ng ml^{-1}		3500 ng ml^{-1}	
Cycle number	ng ml^{-1}	% deviation	ng ml^{-1}	% deviation	ng ml^{-1}	% deviation
0	148	−1.3	1499	−0.1	3524	0.7
1	145	−3.3	1450	−3.3	3505	0.1
2	149	−0.7	1531	2.1	3346	−4.4
3	151	0.7	1515	1.0	3568	1.9

BIRH-414 (internal standard) in methanol was added to each calibration standard, quality control, and unknown sample tube. Solvent was evaporated under a slow stream of nitrogen. Unknown samples, quality control samples, and blank plasma for the calibration standards were vortexed for ≈10 s, and 250 μl of each was quantitatively transferred into the appropriately identified tube. Saline solution (750 μl) was added to each tube and vortexed for ≈10 s. To the tube, 1.5 ml of phosphate buffer (0.1M, pH 2.0) was added and vortexed again for ≈10 s. All tubes were centrifuged to achieve separation of any particulates for ≈10 min at 3000 rpm.

2.5. Solid phase extraction procedure

Using a vacuum manifold, Bond Elut Certify solid phase extraction (SPE) columns were conditioned with 2 ml of methanol, followed by 2 ml phosphate buffer (0.1 M, pH 2). Samples prepared as described above were transferred on to the SPE columns. The loaded SPE columns were washed with 2 ml of acetic acid (1 M), allowed to dry for at least 15 min, and then washed with 6 ml (2 × 3ml) methanol, with about 1 min of drying time in between each wash. The analyte was then eluted with 2 ml of the ammonium hydroxide (2%) in ethyl acetate. The samples were dried down under nitrogen in a 30–35°C water bath, and then reconstituted in 100 μl of mobile phase. Samples were then transferred to autosampler microvials with screw caps and 50 μl of each sample was injected onto the HPLC.

2.6. Analytical data treatment

Chromatography data were acquired by Turbochrom, a hardware and software system developed by PE Nelson. Turbochrom calculated peak height ratios of nevirapine to BIRH-414 for use in linear regression analysis. A weighted (1/Y, where Y = peak height ratio) linear regression was used to determine slope, intercept, and correlation coefficient on each day of analysis. Nevirapine concentrations were calculated from the linear regression and were reported as ng ml^{-1}.

Table 3
Nevirapine long term stability standards prepared on September 4, 1991

Sample age (days)	Date assayed	*n*	50 ng ml^{-1}	% deviation	250 ng ml^{-1}	% deviation	1000 ng ml^{-1}	% deviation	4000 ng ml^{-1}	% deviation
1	9/5/91	1	39	−22	242	−3	909	−9	3514	−12
365	9/3/92	2	53	6	250	0	1011	1	3936	−2
645	6/10/93	2	47	−6	230	−8	932	−7	3675	−8
1084	8/23/94	4	50	0	238	−5	950	−5	3823	−4

Table 4
Nevirapine room temperature stability standards

Nevirapine concentration ng ml^{-1}	n	Thawed prior to analysis		Thawed 4 days		Thawed 21 days	
		Calculated	% deviation	Calculated	% deviation	Calculated	% deviation
100	2	88	−12	97	−3	104	4
3000	2	2894	−4	3000	0	3016	1
7000	2	6675	−5	6716	−4	7139	2

3. Results and discussion

3.1. Linearity and sensitivity

The linearity of extracted nevirapine was determined by spiking blank plasma with known amounts of analyte, in order to obtain concentrations of 25, 50, 100, 250, 500, 1000, 2500, 5000, and 10 000 ng ml^{-1}. The spiked plasma samples were extracted in the manner described above. Over a 2 year period, thirty-six separate calibration curves were produced having an average slope of 0.000401 with a relative standard deviation of 3.19% and an average intercept of 0.001558. The correlation coefficients exceeded 0.9996 in every case. The lower limit of quantitation was determined by the lowest standard included in the standard curve. For 250 μl samples, an LOQ of 25 ng ml^{-1} is ≈ 15 times the absolute limit of the assay, which is defined as three times the chromatographic noise.

3.2. Recovery

Absolute recovery was calculated by comparing the peak response of extracted standards to that of un-extracted standards prepared at the same theoretical concentration. Using this method, the absolute recovery of nevirapine from plasma was found to be 88% or better over the entire range of the standard curve.

3.3. Selectivity and specificity

Nevirapine and the internal standard BIRH 414, can be chromatographically separated on a thermostated (30°C) reversed phase C_8 column. Retention times were ≈ 5.5 min for nevirapine and 7.5 min for BIRH 414. The major metabolite elutes near the void volume and does not interfere in the quantitation of nevirapine.

Specificity was examined for human plasma, serum, milk and cerebrospinal fluid. Up to six separate individual blank samples from each matrix demonstrated that there were no endogenous peaks at the retention time of either nevirapine or BIRH414.

Possible interference by concomitant medications was evaluated in this system by spiking nevirapine quality control samples with the concomitant medication. These spiked samples were then extracted and chromatographed. This procedure was capable of demonstrating not only the lack of chromatographic interference, but also can prove that a concomitant medication had no effect on the extraction or quantitation of nevirapine. Drugs that showed no interference included indinavir, ritonavir, saquinavir, nelfinavir, zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), stavudine (D4T), clarithromycin, rifabutin, and rifampin.

3.4. Precision and accuracy

Intraday precision was evaluated at four analyte concentrations of 100, 2000, 3000, and 7000 ng ml^{-1} using quality control samples. These control samples were chosen to represent conditions similar to expected concentrations of study samples. As shown in Table 1, within run relative standard deviations based on the calculated concentrations ranged from 2.8 to 4.6%. Accuracy was calculated as 107.0, 100.9, 86.9 and 94.6% for the 100, 2000, 3000 and 7000 ng ml^{-1} quality control samples respectively.

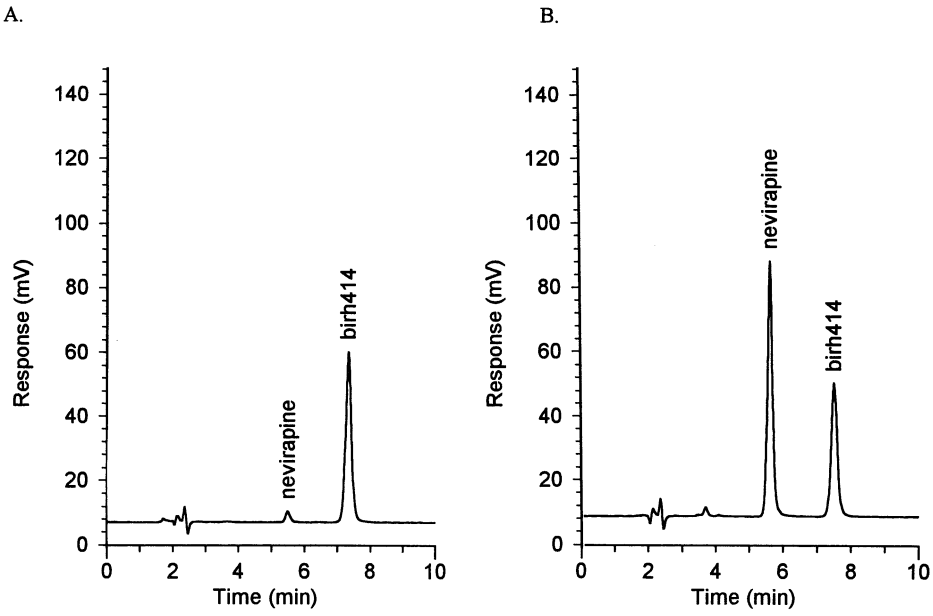

Fig. 2. Representative chromatograms of plasma from human subjects obtained (A) 144 h after a single dose of 200 mg of nevirapine determined to be 158 ng ml^{-1}, and (B) a trough level obtained at steady state on day 32 following 200 mg B.I.D. of nevirapine determined to be 4837 ng ml^{-1}.

In a single study which spanned 2 years, inter-day precision was also evaluated at four analyte concentrations of 100, 2000, 3000, and 7000 ng ml^{-1} using quality control samples. Table 1 shows that the relative standard deviations based on the calculated concentrations ranged from 4.1 to 11.1% with accuracies of 111.3, 96.2, 101.1, and 99.2% for the 100, 2000, 3000, and 7000 ng ml^{-1} quality control samples respectively.

3.5. Stability

Stability of nevirapine in human plasma during repeated freeze–thaw cycles was evaluated for 150, 1500, and 3500 ng ml^{-1} samples. The calculated concentrations after repeated freezing and storage at −20°C were compared to the calculated concentrations of freshly spiked plasma at similar concentrations. The data presented in Table 2 shows that the deviation from nominal did not exceed 5% for any of the nevirapine concentrations.

Long term freezer stability of nevirapine was assessed for 1084 days under normal freezer storage conditions. Stability standards of 50, 250, 1000, and 4000 ng ml^{-1} were prepared in bulk on September 4, 1991, aliquoted into cryotubes and stored below −20°C. After nearly 3 years, each of the concentrations was determined to be within 15% of the nominal concentration (Table 3).

Occasionally clinical nevirapine samples were received that had thawed while in transit. When no duplicate samples were available, it was necessary to analyze the thawed samples. Experiments were performed to demonstrate the effects of such conditions. Table 4 shows the percent deviation from nominal of quality control samples that were analyzed immediately after thawing, as well as quality control samples that were thawed and allowed to stand at room temperature for 4 or 21 days prior to analysis. After being thawed for 21 days, the 100, 3000, and 7000 ng ml^{-1} samples remained within 15% of the nominal concentrations. The 2000 ng ml^{-1} quality control samples were not evaluated under these conditions.

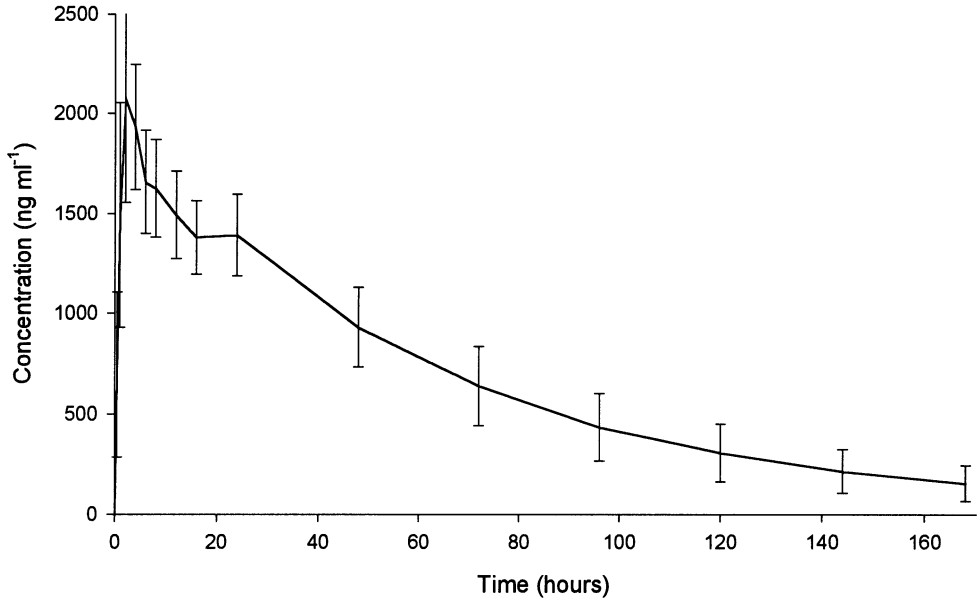

Fig. 3. Plasma concentration–time profile obtained after a single dose of 200 mg of nevirapine in healthy human subjects ($n = 36$).

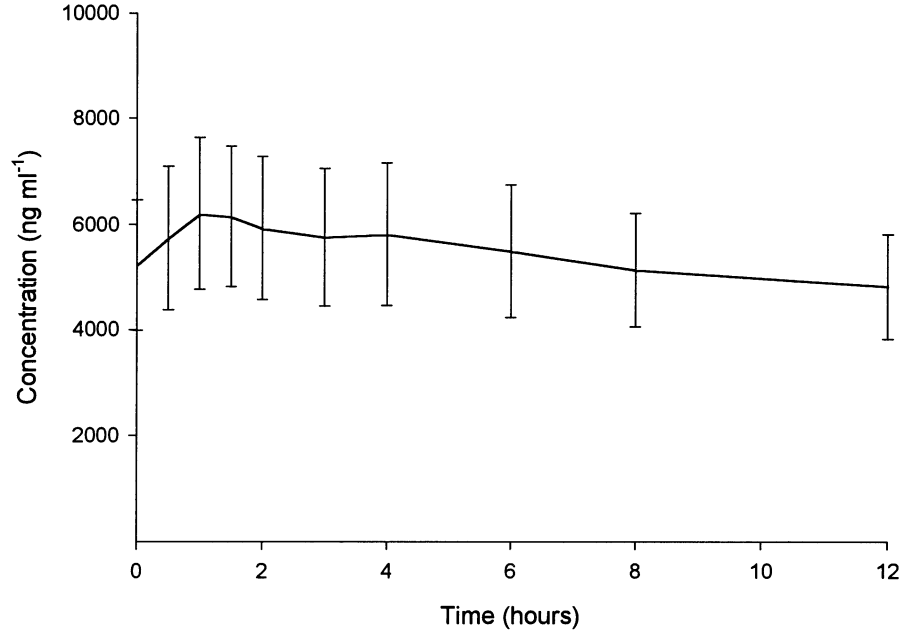

Fig. 4. Plasma concentration–time profile obtained at steady state following dosing of patients with nevirapine at 200 mg B.I.D. ($n = 20$).

3.6. Diverse matrices

This HPLC method was cross validated for the analysis of nevirapine in human serum, milk, and cerebrospinal fluid. The precision of the quality control standards back calculated against the

plasma calibration curve was $\leq 6.44\%$ for the serum quality control standards, $\leq 11.31\%$ for the milk quality control standards, and $\leq 12.96\%$ for the cerebrospinal fluid quality control standards. The accuracy of the quality control standards was $\leq 3.2\%$ for the plasma samples, $\leq 4.9\%$ for the serum samples, $\leq 8.1\%$ for the milk samples, and $\leq 6.6\%$ for the cerebrospinal fluid samples. The average correlation coefficient was 0.9998 for plasma ($n = 6$), 0.9987 for serum ($n = 6$), 0.9997 for milk ($n = 6$), and 0.9998 for cerebrospinal fluid ($n = 3$).

3.7. Applications

Demonstration of the applicability of this method is shown in Figs. 2–4. The method as validated allows for its use in both single and multiple dose study designs. Fig. 1A is a chromatogram of human plasma from one subject 144 h after a single 200 mg dose of nevirapine. Fig. 1B is a chromatogram of a plasma trough level obtained from a patient at steady state following 200 mg B.I.D. of nevirapine. In a single dose study of healthy volunteers, the range of the method allowed for nevirapine plasma concentration determinations out to 168 h post dose (Fig. 2). The plasma concentration–time profile obtained at steady state following dosing of patients with nevirapine at 200 mg B.I.D. in Fig. 3 shows that all plasma levels were well within the range (25–10 000 ng ml^{-1}) of the method.

4. Conclusion

The HPLC method provides a sensitive, precise, and specific means for the quantitation of nevirapine in human plasma, serum, milk, and cerebrospinal fluid. This method has been used to successfully analyze over 25 000 samples in support of phase I, II, III and IV clinical programs for nevirapine.

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