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Determination of nevirapine, an HIV-1 non-nucleoside reverse transcriptase inhibitor, in human plasma by reversed-phase high-performance liquid chromatography

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Abstract

A sensitive and rapid high-performance liquid chromatography method has been developed to measure the levels of the HIV-1 non-nucleoside reverse transcriptase inhibitor nevirapine in human plasma. The sample pre-treatment consists of a protein precipitation with perchloric acid. A Hypersil ODS column is used at ambient temperature and a wavelength of 280 nm is used for ultraviolet detection. The mobile phase contains acetonitrile and a 60 m*M* phosphate buffer pH 4.5 (30:70, v/v). The detection limit of the method is 0.05 mg/l using 150 μl of plasma. The lower and upper limit of quantitation are 0.1 mg/l and 10 mg/l, respectively. The average recovery of nevirapine is 101.8% with a variation of 4.6%. The average inter-assay precision is 2.4%, the average intra-assay precision 2.9% and the average accuracy 97%.

Keywords: Nevirapine

1. Introduction

In the battle against AIDS, many drugs have been tested on their potential of inhibiting HIV-1 replication. Nowadays combination therapy existing of two nucleoside reverse transcriptase inhibitors and one protease inhibitor is recommended. Recently, nevirapine, a representative of a new class of antiretroviral drugs, the non-nucleoside reverse transcriptase inhibitors, has been introduced.

Nevirapine, 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido- [3,2-b:2′,3′-e][1,4]diazepin-6-one, a dipyridodiazepinone developed by Boehringer Ingelheim Pharmaceuticals, (during its development also refered to as BI-RG-587), was the first non-nucleoside reverse transcriptase inhibitor to be approved by the FDA and also the first to be used in clinical trials in the Netherlands.

Several studies have described the pharmacokinetics of nevirapine [1–3]. The drug is completely absorbed and widely distributed throughout

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body tissues and the central nervous system. It has a volume of distribution of 1.37 l/kg. Peak concentrations can be found after 2.2 h. Nevirapine has a half-life in the range of 20 to 84 h.

Montaner et al. showed that a maximum virological effect was found after 16 weeks in a triple drug therapy using zidovudine, didanosine and nevirapine [4]. 68% Of the patients using the triple drug therapy showed at that time a plasma HIV-1 RNA level below 20 copies per milimeter.

At the University Medical Center St Radboud, Nijmegen, The Netherlands, nevirapine was selected for determination and quantification in patient samples and for future clinical pharmacokinetic research. Hence an analytical method had to be developed to determine nevirapine in human plasma at therapeutic concentrations. The method has been validated conform local standard operating procedures.

2. Materials and methods

2.1. Chemicals

Nevirapine was obtained from Boehringer–Ingelheim Pharmaceuticals (Ridgefield, Connecticut, USA) Acetonitrile and Methanol HPLC Quality were obtained from Labscan Analytical Sciences (Dublin, Ireland). All other reagents were purchased from Merck (Darmstadt, Germany) and were all of the pro analysi quality.

Throughout the development of the assay demineralized water was used. Blank human plasma was obtained from the Blood Transfusion Service Nijmegen, the Netherlands. The drugs used for investigation for possible interference with the method being developed, were acquired from Sigma Chemical Co. (St. Louis, MO, USA) or were extracted out of commercial products by dissolving the compounds in a relevant organic solvents.

2.2. Stock solutions and plasma samples

The nevirapine stock solution (1 g/l) was prepared by dissolving the drug in dimethylsulfoxide (DMSO). The stock solutions used for calibration lines were gained from a different stock solution as those used for the quality controls. All stock solutions were preserved at a temperature of −20°C. Blank human plasma and patient plasma were preserved at −20°C.

2.3. Equipment

A Hypersil ODS 5 μm, 250×4 mm, analytical column (Alltech, No. 288216) was protected by a reversed-phase 75×2.1 mm guard column (Chrompack, No. 28603). The high-performance liquid chromatography system consisted of a Spectra Physics SP8800 gradient ternary pump, a Spectra Physics SP4290 integrator, a Spectra Physics SP8775 autosampler, a Gastorr GT-103 degasser and a Kratos Analytical spectroflow 783 Ultraviolet detector. The ultraviolet spectra of nevirapine in methanol were measured with a Perkin-Elmer Lambda2 UV–VIS spectrophotometer.

2.4. Sample pretreatment

In order to obtain the first standard sample of nevirapine, 10 μl of the nevirapine stock solution in DMSO was added to 990 μl of blank human plasma in a 1.5 ml Eppendorf micro test tube in duplicate. In addition, quantities of 300, 100, 30 and 10 μl of the first standard sample were added to successively 700, 900, 970 and 990 μl blank human plasma. Hence the nevirapine calibration concentrations were 10, 3, 1, 0.3 and 0.1 mg/l, respectively.

Each micro test tube was briefly vortexed and subsequently 150 μl of the contents of each micro test tube was added into separate empty micro test tubes. Correspondingly, 150 μl of 0.33 *M* perchloric acid was added for protein precipitation. The micro test tubes were again briefly vortexed and centrifuged at 11 000 *g* for 5 min. Two hundred μl of the supernatant was added into a 200 μl autosampler vial insert, ready for injection into the high-performance liquid chromatography system.

2.5. Chromatography

An aliquot of 100 μl was injected onto the column. The mobile phase consisted of acetonitrile and a 60 m*M* potassium phosphate buffer pH 4.5 (30:70 v/v). The flow-rate was maintained at 1.5 ml/min.The analytes were detected by UV detection

at a wavelength of 280 nm with a run time of 6.5 min. The Absorption Unit Full Scale (A.U.F.S.) was set at 1.0.

2.6. Validation procedures

The specificity of the analytical method was determined by analysing possible interference of endogenous compounds of blank human plasma by using six blank human plasma samples, each from different individuals who did not use nevirapine.

Subsequently a large number of drugs and metabolites were analysed for their ability to interfere with nevirapine. The metabolites of nevirapine have not been analysed since they were not obtainable.

The limit of detection was determined as the lowest concentration that could be reliably distinguished from background noise levels in six blank human plasma samples. The difference between a sample containing nevirapine and a blank sample was tested with a paired *t*-test. If $p<0.05$ the test was considered significant. Concentrations that gave a signal-to-noise ratio of 3 were first selected to set the limit of detection.

The lower limit of quantitation was determined by producing the desired concentration in fivefold. It was a requisite that the relative standard deviation and percentual deviation of this concentration were both less than 20%.

The accuracy and precision of the developed method were determined by analysis of three quality control samples containing a low, middle and high concentration of nevirapine, respectively 0.191, 0.787 and 4.92 mg/l. Each of the quality control samples was produced in fivefold and analysed in three separate runs. Subsequently, the mean of each set of concentrations and the percentual deviation of the quality control samples were calculated. The accuracy was calculated as the average percentage of the nominal concentration. One-way analysis of variance (one-way ANOVA), with the day of analysis as variable of classification, was used to calculate the inter- and intra-assay variation.

The following formulas were used in order to calculate the inter-assay precision and intra-assay precision respectively:

$$\frac{\{(\text{Day mean square} - \text{Error mean square})/n\}^{1/2}}{\text{Grand mean}} \times 100\%$$

$$\frac{(\text{Error mean square})^{1/2}}{\text{Grand mean}} \times 100\%$$

The Day mean square, the Error mean square and Grand mean are expressions originating from ANOVA. *n* is the number of replicates within each day (five) for each concentration. If the Error mean square is higher than the Day mean square, the inter-assay precision is regarded as zero. This signifies that no significant additional variation is observed as a result of performing the assay in different runs.

The recovery of nevirapine was determined by generating three calibration curves in duplicate in plasma and generating three calibration curves in demineralised water, hence without protein precipitation. By dividing the response of nevirapine of the calibration curve generated in water by the response of nevirapine of the calibration curve generated in plasma a quotient could be determined. By averaging this quotient the recovery is obtained.

2.7. Stability

Stability of nevirapine was evaluated with regard to various conditions. The stability in plasma after precipitation was examined for 13 h at ambient temperature. Subsequently stability tests of nevirapine were performed in plasma and in whole blood for the total duration of 164 h both at ambient temperature and 4°C with a nevirapine concentration of 4.444 and 0.572 mg/l in duplicate. Also, tests in plasma were performed by keeping duplicates of the quality control samples and stock solution at temperatures of −20°C for various periods of time.

Additionally, by freezing the two highest quality control samples at −20°C and subsequently thawing these samples by keeping them at ambient temperature, and performing this cycle two times in a row, the freeze–thaw effect was studied.

2.8. Pharmacokinetic experiments

One HIV-infected person gave informed consent to participate in a clinical pharmacokinetic experiment that had the approval of the local Commitee of Ethics of the University Hospital Nijmegen.

Plasma of that person was collected from 0 to 12

h, after ingestion of 200 mg of nevirapine (one tablet of Viramune 200 mg). The daily dosage was 200 mg every 12 h. Pharmacokinetic analysis was performed by noncompartemental methods.

3. Results

3.1. Development phase

In order to acquire the best suitable wavelength for the detection of nevirapine by UV-absorption, a UV-spectrum was established. The UV-spectrum revealed that maximum absorbance peaks could be detected at 205, 216 and 280 nm. Because the majority of the drugs tested in this assay for the determination of interference with nevirapine were likely to have maximum absorbance peaks at lower, non-specific, wavelengths than 280 nm, it was decided that 280 nm was the most preferred wavelength. Nevirapine has a lower molar absorbance coefficient at 280 nm than at 205 or 216 nm, but considering the fact that therapeutic concentrations of nevirapine are seen in the mg/l range and not in the μg/l range [1], it was anticipated that detection at 280 nm was sensitive enough for the determination of nevirapine.

An appropriate proportion of acetonitrile and water in the mobile phase had to be found for the separation of blank plasma interference peaks with the nevirapine peak. A suitable separation was found with a mobile phase consisting of acetonitrile:water (30:70 v/v). The water-phase, chosen on prior experience grounds, consisted of a 60 m*M* phosphate buffer and was maintained at that concentration.

The optimal pH of the phosphate buffer appeared to be 4.5. At higher pH more interference from co-medication was observed, while at lower pH the nevirapine peak tailed. While sulfamethoxazole showed possible interference with nevirapine at pH 4.5, at therapeutic levels of sulfamethoxazole (100 mg/l) no interference of sulfamethoxazole was seen with regard to nevirapine.

The standard curves that were produced first resulted in inaccurate determination of the lowest concentration point. This problem was resolved by saturating the plastic pipette-tip through draining of stock solutions three times prior to addition to plasma.

To investigate the effects of protein precipitation, aliquots of 150 μl acetonitrile, trichloroacetic acid and perchloric acid were added to 150 μl of plasma containing nevirapine and compared with regard to recovery of nevirapine and position of the nevirapine peak on the chromatograms. Perchloric acid was preferable to acetonitrile and trichloroacetic acid since it resulted in the largest recovery of nevirapine without interference. The retention time of nevirapine at given circumstances was 3.9 min (intra-day standard deviation: 1.4%). Representative chromatograms of blank human plasma, blank human plasma spiked with 0.282 mg/l nevirapine, and plasma from an HIV-infected patient spiked with 0.282 mg/l nevirapine, are shown in Fig. 1A–1C, respectively.

3.2. Validation phase

Drugs frequently used by HIV-1-infected patients were analysed regarding their capability of interfer-

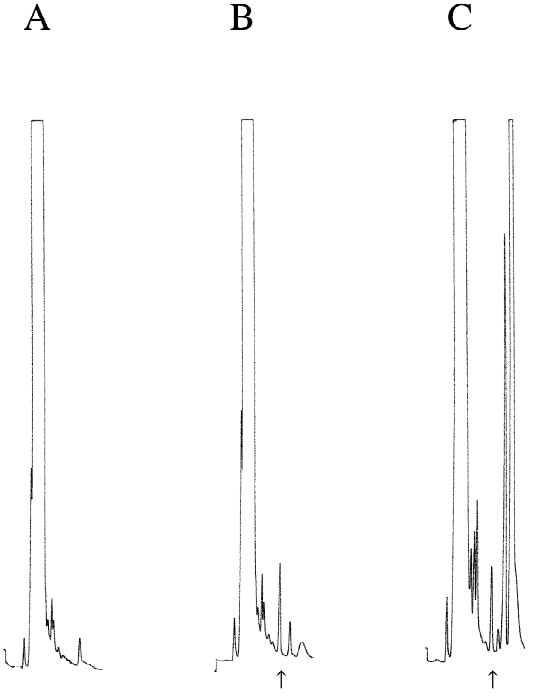

Fig. 1. Representative chromatograms of blank human plasma (A), blank human plasma spiked with 0.282 mg/l nevirapine (B) or plasma from an HIV-infected patient spiked with 0.282 mg/l nevirapine (C). The arrows indicate the nevirapine peak.

Table 1
Inter- and intra-day precision and accuracy

Concentration (mg/l)	Inter-assay precision (%)	Intra-assay Precision (%)	Accuracy (%)
0.191	2.4	3.4	96.9
0.765	3.0	3.9	91.5
4.78	1.9	1.3	102.6

ing with nevirapine. The drugs analysed were: acyclovir, amphotericine B, amoxicillin, atovaquone, calcium folinate, clarithromycin, clindamycin, clofazimin, caffine, dapsone, didanosine, erythromycin, ethambutol, famotidine, fluconazole, folic acid, ganciclovir, indinavir, isoniazid, itraconazole, ketoconazole, lamivudine, methadone, nelfinavir, oxazepam, paracetamol (acetaminophen), pentamidine, phenytoin, pyrazinamide, pyrimethamine, rifabutin, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, sulfametrol, trimethoprim, zalcitabine, zidovudine. Additionally the metabolites mono-acetyldapson, N_4-acetylsulfamethoxazole, N_4-hydroxy-sulfamethoxazole and N_4-acetylsulfametrol have been analysed on similar grounds. None of the drugs or metabolites showed any interference with the analytical method developed except for ofloxacin. Ofloxacin had a retention time of 3.7 min and interfered at therapeutic concentrations with the nevirapine-peak. However, ofloxacin is a drug rarely used by HIV-1-positive persons in our hospital. Less than 0.5% of all HIV-1 positive patient samples received during the past 2 years contained ofloxacin.

The detection limit was 0.05 mg/l using 150 μl of plasma. The lower limit of quantification was 0.1 mg/l. Former studies [1] indicated that therapeutic concentrations of nevirapine are to be expected above 1 mg/l.

The data on precision and accuracy of the assay are listed in Table 1. The average recovery of the three separate runs was 101.8% with a variation of 4.6%.

Table 3
Stability (% of concentration at T=0) of nevirapine in plasma at −20°C after various periods of storage

Time (months)	Concentration (mg/l)	
	0.787	4.92
2	93.9	97.1
3	85.6	92.5
6	99.7	99.8
9	98.3	96.9

The nevirapine concentrations used for the creation of the calibration curve were calculated through linear regression after the data had been logarithmically converted. This conversion was done with the objective of giving each concentration of the calibration curve equal significance. An example of the equation of the calibration line is: $log\ [peak\ height] = 0.987 \times log\ [nevirapine\ concentration] + 3.703$. Linearity of this model was demonstrated by the fact that the X-coefficient approximates 1 and by the *F*-test for lack of fit. The sum of the % deviation from nominal concentrations in the calibration curve varied between 5 and 10%, indicating an average deviation of 1–2% per calibration sample.

Results of the stability tests performed, are shown in Tables 2 and 3. Stock solutions of nevirapine dissolved in methanol seemed less stable than nevirapine dissolved in DMSO. The average stability of nevirapine after precipitation examined for 13 h at ambient temperature was 99% with a percentual deviation of 1.0%.

After about 9 months of storage, there was no significant decrease of nevirapine concentrations the quality control samples. The outcome of the freeze–thaw effect was that the high quality control concentrations measured had an average percentual

Table 2
Stability of nevirapine in plasma and whole blood at ambient temperature and 4°C

Time (h)	Plasma at ambient temperature (%)	Plasma at 4°C (%)	Whole blood at ambient temperature (%)	Whole blood at 4°C (%)
24	103.6	101.4	105.4	105.4
96	104.4	101.1	101.7	101.8
164	99.1	98.9	112.3	98.6

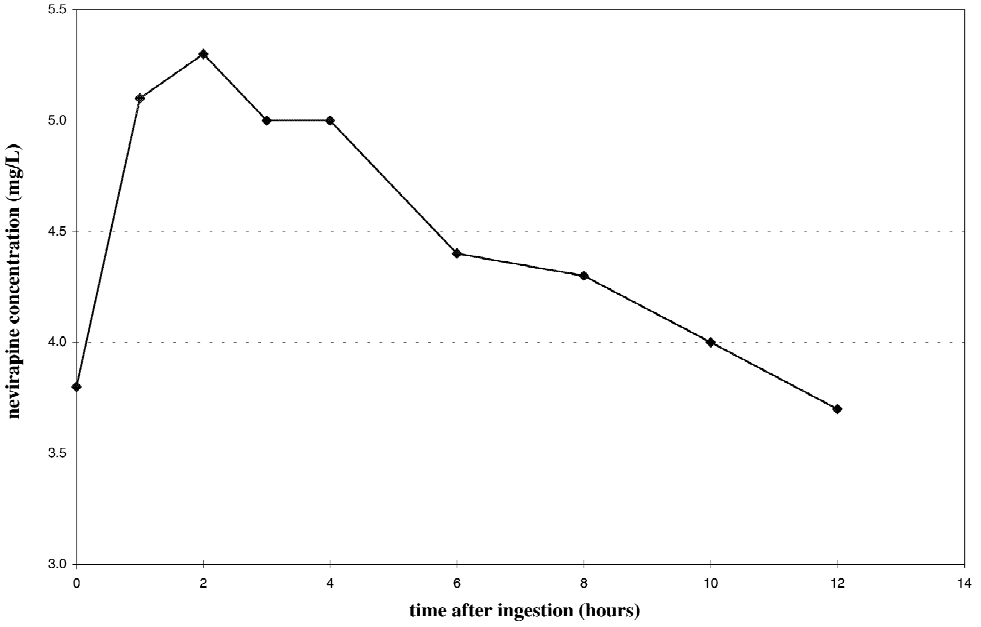

Fig. 2. Pharmacokinetic curve of nevirapine after ingestion of a 200 mg dose in a single HIV-infected person.

deviation of 2.8% with regard to the nominal concentration. Likewise the percentual deviation in the middle quality control sample was 13.1%.

3.3. Pharmacokinetic experiment

The nevirapine plasma concentration–time profile of an HIV-1-infected person using nevirapine chronically at a dose of 200 mg every 12 h was analysed (Fig. 2). The pharmacokinetic parameters were derived from this curve and compared with available literature values also shown in Table 4. This table also includes the most relevant patient characteristics. Our values were similar to the values found in literature.

4. Discussion and conclusion

During the development of this assay an attempt was made to find a simple, rapid and sensitive high-performance liquid chromatography method for the detection of nevirapine in human plasma. After examining literature, only one abstract of an article was found that dealt with this subject. The article was published by Jayaraj et al., investigators from the manufacturer of nevirapine, Boehringher-Ingelheim Pharmaceuticals [5].

In contrast to that method, our method does not contain an ion-pair reagent nor an internal standard, which makes our method an easier one. Additionally, through our method, a more clinical relevant range of plasma nevirapine concentrations was obtained that even extends that of Jayaraj et al. Furthermore, a higher recovery is gained since no comprehensive means of extraction were used. Especially the 280 nm UV-wavelength worked in our advantage with regard to interference by drugs frequently taken by HIV-1 positive persons in combination with nevirapine.

Shortly after we had finished the validation protocol and this manuscript was in preparation, two other methods for the determination of nevirapine were published [7,8]. The method of Van Heeswijk et al. [7] method resembles our method with regard to detection wavelength and sensitivity. Differences can be found in sample pretreatment, mobile phase, and a shorter run-time in our method; furthermore, our stability data are more extensive than those of Van Heeswijk et al., [7]. The method of Pav et al., [8]

Table 4
Pharmacokinetic experiment of an HIV-1 positive person using nevirapine

Patient characteristics		
Gender (M/F)	F	
Age (year)	26	
Weight (kg)	71	
Dose of nevirapine (mg)	200	
Co-medication	Zidovudine Lamivudine	
Pharmacokinetic Parameters		**Reference values [3,6]**
T_{max} (h)	2.00	2.2
C_{max} (mg/l)	5.30	
C_{min} (mg/l)	3.70	
AUC_{0-12} (h mg/l)	53.9	
CL/F (l/h)	3.96	
CL/F. kg (l/h kg)	0.0557	0.0533
Vd/F (l)	134	
Vd/F. kg (l/kg)	1.88	1.37, 1.17
$T_{1/2}$ (h)	23.4	22–84

differs from ours in that in their method uses a solid-phase extraction procedure as sample pretreatment. The stability data reported by them confirms our data.

Although only one HIV-1 positive person is mentioned here, it is seen that the calculated pharmacokinetic parameters of the HIV-1-positive person are similar to those published in literature [1–3] and hence support that our method is a liable one for the detection and determination of nevirapine plasma concentrations of HIV-infected patients.

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