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Rapid determination of nevirapine in human plasma by gas chromatography

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Abstract

A sensitive and rapid gas chromatographic method has been developed to determine the levels of the HIV-1 non-nucleoside reverse transcriptase inhibitor nevirapine in human plasma. Quantitative recovery following liquid–liquid-extraction with diethylether from 500 μl of human plasma was achieved. Subsequently, the assay was performed with a CP-Sil 5CB capillary column, 15 m×0.32 mm×1.0 μm film thickness with a nitrogen–phosphorous-detector (NPD), Helium 5.0 was used as carrier gas with a constant inlet pressure of 7 p.s.i. Linear standard curves were obtained for concentrations ranging from 10 to 20 000 ng/ml. The calculated intra- and inter-day coefficients of variation were below 8%.

Keywords: Nevirapine

1. Introduction

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1), currently for the treatment of HIV-1 infected adults and children [1]. NNRTI-based regimens for antiretroviral therapy may have several advantages including more convenient administration regimens, lower tablet volume and avoiding PI-related metabolic disturbances. Long-term efficacy of NVP-containing regimens was shown in multiple clinical studies [2,3]. NVP is principally metabolized by CPY3A4 and CYP2B6. It has not only potential to induce its own metabolism but it is also involved in drug interactions with several therapeutic classes [4]. Several high-performance liquid chromatographic (HPLC) assays with ultraviolet detection for the quantitative determination of NVP in plasma have been described [5–8]. High-performance liquid chromatographic methods for the simultaneous determination of HIV-1 protease inhibitors and the HIV-1 non-nucleoside reverse transcriptase inhibitor efavirenz are suitable for routine clinical analysis in our laboratory [9,10]. The method presented here

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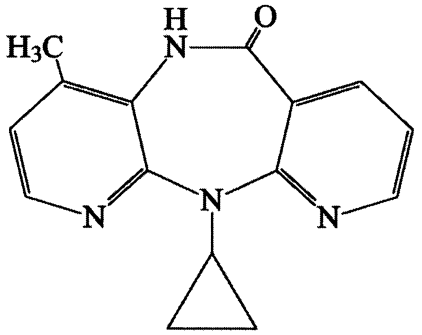

Fig. 1. Molecular structure of nevirapine.

describes the validation of a gas chromatographic method with NP-detection for the determination of NVP concentrations in human plasma during antiretroviral therapy in patients with HIV infection.

2. Experimental

2.1. Chemicals and reagents

Nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2',3'-e]diazepin-6-one) (Fig. 1) was kindly provided by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA). The internal standard Scandicaine 1% was purchased from Astra Chemicals (Astra GmbH, Wedel, Germany) (Fig. 2).

Diethyl ether, dimethyl sulfoxide, methanol, distilled water, sodium dihydrogen carbonate, sodium carbonate were obtained in analytical grade purity from Merck (Darmstadt, Germany).

Blank, drug-free plasma was obtained from Sigma–Aldrich Chemie (Deisenhofen, Germany).

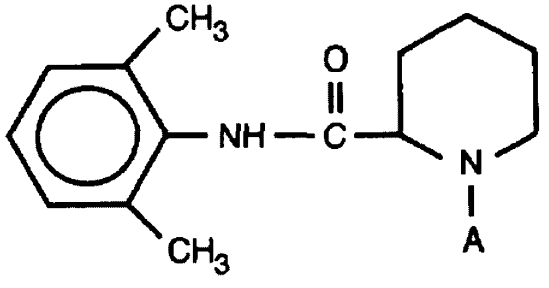

Fig. 2. Molecular structure of scandicaine.

2.2. Chromatographic equipment and conditions

A Hewlett-Packard 5890 series II gas chromatograph consisting of a 7673 autosampler and a HP 3396 series II integrator (Hewlett-Packard GmbH, Waldbronn, Germany), equipped with a nitrogen–phosphorus-detector (NPD), were used for gas chromatographic analysis. Chromatography was performed with a CP-Sil 5CB capillary column, 15 m×0.32 mm×1.0 μm film thickness (Chrompack GmbH, Frankfurt/Main, Germany). Helium 5.0 was used as carrier gas with a constant inlet pressure of 7 p.s.i. The injection mode was splitless (1 μl) for 0.30 min. The following temperature program was applied: 0 min at 210°C, then 10° per min up to 310°C, the final temperature 310°C was held for 2 min.

The temperature of the injection port and the detector (NPD) were set at 300°C.

2.3. Standard preparation

The initial stock solution of nevirapine (5 mg/100 ml) was prepared in a mixture of methanol/dimethyl sulfoxide (1:1, v/v) as described previously [5]. This solution was appropriately diluted for the preparation of working solutions at concentrations of 10–20 000 ng/ml.

A stock solution of the internal standard (scandicaine) was prepared in methanol to yield a final concentration of 10 mg/100 ml. Each solution was stored at 4°C and was stable for at least 3 months. For preparation of the plasma standard samples an appropriate amount of the working solutions and the internal standard (3000 ng/ml) were added to blank plasma to achieve the mentioned range of calibration concentrations.

2.4. Sample preparation

A 500-μl aliquot of plasma was mixed with the same amount of carbonate buffer (0.1 *M* sodium carbonate/sodium hydrogencarbonate (1.8:8.2), pH 9.4), furthermore a 75-μl aliquot of solution of internal standard (Scandicaine) was added to a 10-ml glass tube. After vortexing for 10 s, the tubes were capped and extracted twice with 3 ml diethyl ether for 30 s, followed by centrifugation at 3000 *g* (4°C). The organic layers were transferred to a glass

centrifuge tube and evaporated to dryness with a gentle stream of nitrogen at 37°C.

The residue was reconstituted in 200 μl methanol/dimethyl sulfoxide (1:1, v/v). The organic layer was transferred to autosampler vials with glass micro inserts for GC analysis.

2.5. Preparation of quality control samples

A quality control (QC) stock solution was prepared by dissolving 2.5 mg Nevirapine in 50 ml of a mixture of methanol/dimethyl sulfoxide (1:1, v/v). Subsequent QC stock samples were prepared at the 750, 2500 and 5500 ng/ml level by serial dilution with blank, drug-free plasma.

On each validation day, working QC samples were prepared at each level by the same procedure as the sample preparation described above.

2.6. Specificity and selectivity

In order to evaluate levels of endogenous compounds with potential for interference with the analytical method, analysis of six different blank samples was performed. The following compounds were determined: abacavir, amprenavir, didanosine, efavirenz, fluconazole, folinic acid, ganciclovir, itraconazole, lamivudine, methadone, methotrexate, nelfinavir, M8-metabolite of nelfinavir, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, zalcitabine, and zidovudine. Three spiked plasma samples were analysed for each substrate.

2.7. Limit of detection

The limit of detection in plasma (LOD) was defined by the lowest detectable concentration yielding a signal-to-noise ratio of three, indicating a significant difference of spiked and blank samples in plasma samples of three individuals as determined by the two-tailed, paired Student's *t*-test.

2.8. Limit of quantitation

For the concentration to be accepted as the lower limit of quantitation (LLQ) the measure of accuracy (percent deviation from the nominal concentration) and precision (relative standard deviation) are to be less than 20%. All samples were assayed in triplicate.

The upper limit of quantitation (ULQ) was arbitrarily set at 20 000 ng/ml.

2.9. Accuracy, precision, linearity and recovery

Intra-day accuracy and precision of the method were determined by measuring six replicate QC samples at three different concentrations of nevirapine (750, 2500 and 5500 ng/ml).

To obtain the inter-day accuracy and precision, 18 samples of each concentration were analyzed at nine different days as described above.

Accuracy was calculated as the relative error of the nominal concentration. Precision was expressed in terms of relative standard deviation and obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Daily standard curves were evaluated by duplicate analysis of nine spiked plasma samples for nevirapine in the range of 10–20 000 ng/ml.

A linear weighted [1/concentration squared] least squares regression analysis to plot the observed peak area/internal standard ratio of nevirapine was performed. Linearity and assay reproducibility were determined by measuring the standard concentration in five separate assay runs on five separate days.

The linearity of five calibration curves was tested with the *F*-test for lack of fit, using a weight factor of [1/conc].

The recovery of nevirapine in the extraction procedure was determined comparing the detected concentrations of nevirapine (100, 500, 5000 and 10 000 ng/ml) in three extracted spiked plasma samples to those of non-processed standard solutions.

2.10. Analysis of patient samples

Plasma samples derived from 12 HIV-infected patients during antiretroviral therapy with combination of different NRTI zidovudine, lamivudine, stavudine or didanosine, and/or protease inhibitor and nevirapine (200 mg bid) were taken 12 h after the ingestion of 200 mg nevirapine. A total of $n=66$

plasma samples from all patients were obtained by a standardized procedure. Plasma was separated by centrifugation at 3000 *g* for 10 min at 4°C and was immediately stored at −20°C until further analysis.

2.11. Calculation and data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 7.5.2.dt (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Chromatography and detection

Sample preparation by protein precipitation with acetonitrile was described previously [5]. We used a 500-μl aliquot of plasma following liquid–liquid extraction with diethyl ether. So an equal baseline and good sensitivity of our assay was reached. Liquid–liquid extraction further resulted in less residue in the column after a run allowing the highest possible number of runs in one column. The use of the internal standard (ISTD) Scandicaine is simple and makes our assay more reliable than a previously described method without an ISTD [6]. Peak shape, separation from endogenous compounds and separation from other antiretrovirals were therefore optimized using a CP-Sil 5CB capillary column, 15 m×0.32×1.0 μm film thickness (Chrompack GmbH, Frankfurt/Main, Germany).

A chromatogram of a blank plasma sample (Fig. 3) showed no interfering endogenous peaks. A representative GC run of a of a blank human plasma sample spiked with internal standard and nevirapine 73 ng/ml is shown in Fig. 4.

A chromatogram of a plasma sample derived from a patient receiving a PI-sparing therapeutic regimen consisting of zidovudine, zalciabine and nevirapine is shown in Fig. 5. The retention time of nevirapine was 7.02 min. A plasma concentration of 3506 ng/ml was detected.

3.2. Specificity and selectivity

Drug-free plasma samples obtained from healthy individuals were devoid of interference near the retention time of nevirapine and the internal standard.

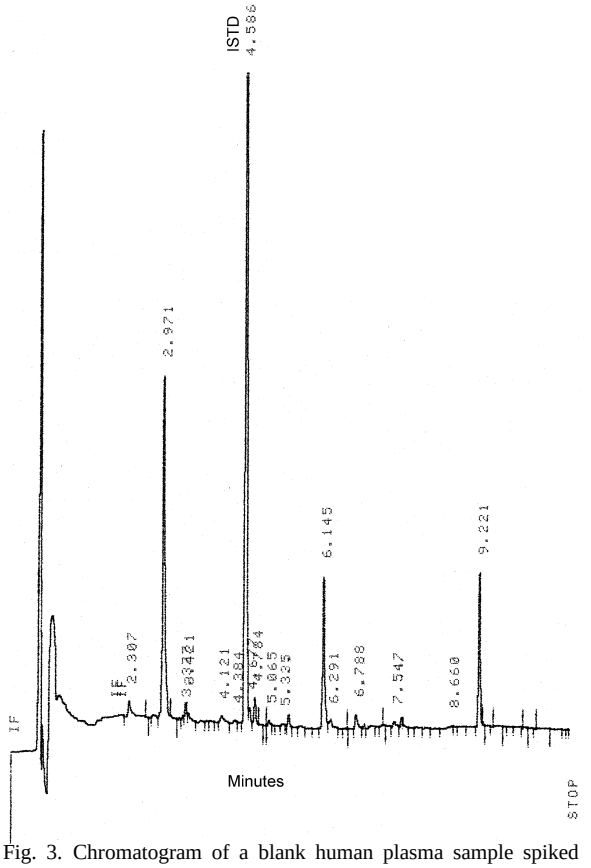

Fig. 3. Chromatogram of a blank human plasma sample spiked with internal standard (RT 4.59 min).

The analysis of plasma samples containing abacavir, amprenavir, didanosine, efavirenz, folinic acid, fluconazole, ganciclovir, itraconazole, lamivudine, methadone, methotrexate, nelfinavir, M8 metabolite of nelfinavir, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, zalcitabine, or zidovudine showed no interference either with the extraction procedure or with the analytical method.

3.3. Limit of quantitation and detection

The detection limit of nevirapine in plasma was determined at 2 ng/ml. The lower limit of quantitation was reached at a concentration of 10 ng/ml. The upper limit of quantitation was arbitrarily set at 20 000 ng/ml.

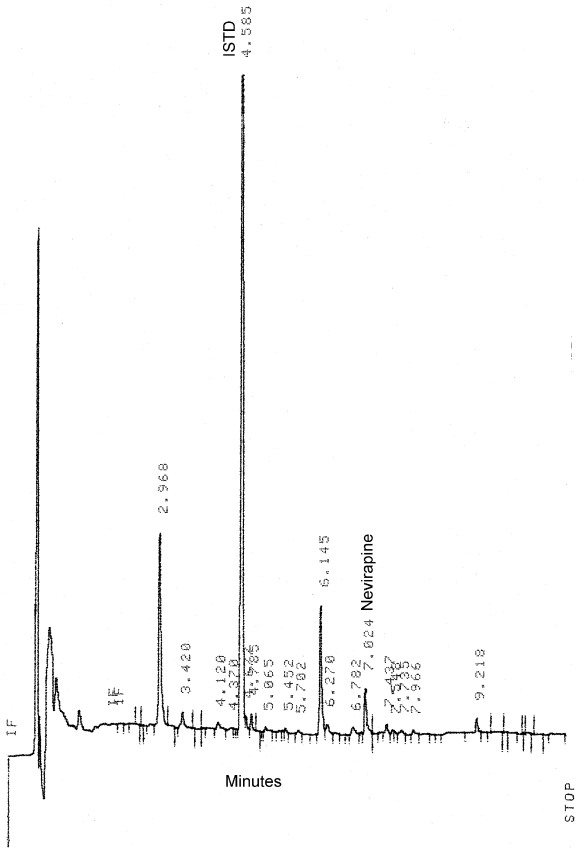

Fig. 4. Chromatogram of a blank human plasma sample spiked with internal standard and nevirapine 73 ng/ml (RT 7.02 min).

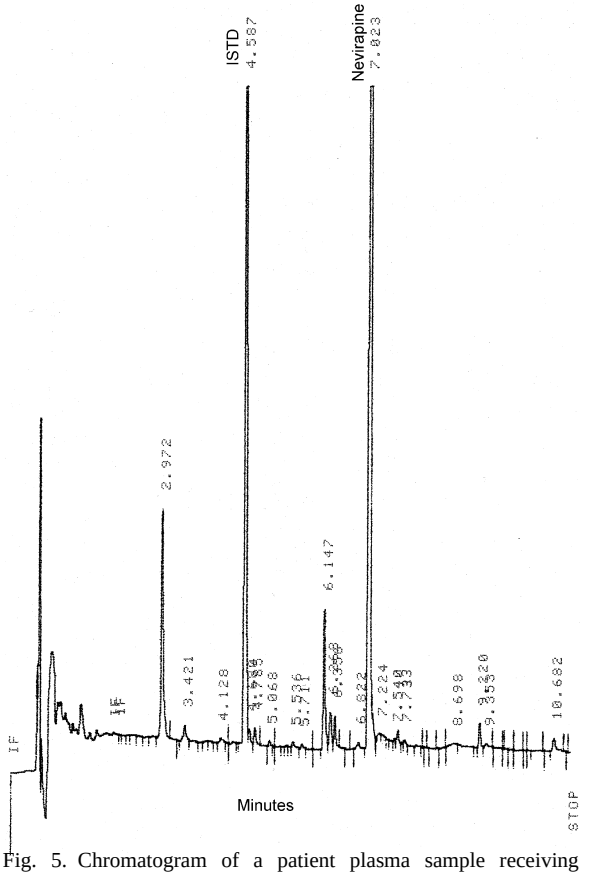

Fig. 5. Chromatogram of a patient plasma sample receiving zidovudine, zalcitabine and nevirapine. A nevirapine concentration of 3506 ng/ml was detected.

3.4. Accuracy, precision, linearity and recovery of the assay

The intra-day accuracy and precision of the method was determined in six analytical runs including three different concentrations. Precision ranged from 1.9 to 3.4%. Intra-day accuracy was 4.8 to 7.3%. The results of intra-day validation are presented in Table 1. The inter-day accuracy and precision of nevirapine as shown in Table 2 were below 10%.

Using the ratios of the observed peak areas for nevirapine and the internal standard in nine spiked plasma samples analyzed in duplicate, the standard curves showed a correlation coefficient of 0.999 (range 10–20 000 ng/ml) as determined by least-square analysis. All calibration curves proved to be linear in the respective ranges listed above in the F-test for lack of fit, which was performed to assess the linearity of the regression model.

The recovery of nevirapine was estimated by comparison of peak areas in extracted spiked drug-free plasma with those of non processed standard solutions. Recovery was found to be 97.8±3.8% (n=3) for nevirapine and 91.7±3.2% for the ISTD, respectively.

Table 1
Accuracy and precision for the analysis of nevirapine in spiked plasma samples: intra-day precision (C.V.%) and accuracy (R.E.%)

Concentration found (ng/ml)	Sample concentration (ng/ml)		
	750	2500	5500
Mean	695	2379	5140
±SD	16.4	46.1	175.6
C.V. (%)	2.4	1.9	3.4
R.E. (%)	7.3	4.8	6.5
n	8	8	8

Table 2
Accuracy and precision for the analysis of nevirapine in spiked plasma samples: Inter-day precision (C.V.%) and accuracy (R.E.%)

Concentration found (ng/ml)	Sample concentration (ng/ml)		
	750	2500	5500
Mean	700	2370	5159
±SD	33.4	129.2	230.4
C.V. (%)	4.8	4.6	4.1
R.E. (%)	6.6	5.1	6.2
n	18	18	18

Stability studies show no degradation of nevirapine (Table 3).

3.5. Analysis of patient samples

Plasma samples derived from 12 HIV-infected patients during antiretroviral therapy with combination of different NRTI zidovudine, lamivudine, stavudine or didanosine and/or protease inhibitor and nevirapine (200 mg bid) were taken 12 h after the last ingestion of 200 mg nevirapine.

Table 3
Stability of nevirapine in spiked human plasma samples

Storage conditions	Conc. (ng/ml)	Recovery (%)	C.V. (%)	*n*
60 min at 56°C	750	103.8	1.7	4
	5500	103.3	1.6	4
24 h at 24°C	750	104.0	1.4	4
	5500	102.8	1.9	4
7 days at 4°C	750	103.0	1.4	4
	5500	97.8	1.3	4
30 days at −20°C	750	103.2	1.3	4
	5500	99.5	0.7	4
Four freeze–thaw cycles	750	99.3	0.7	4
	5500	95.3	2.8	4

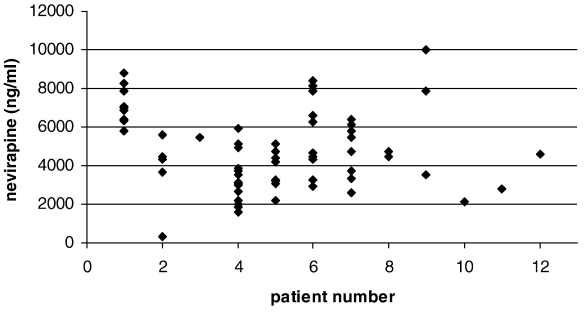

Fig. 6. Nevirapine plasma concentrations (trough levels) from 12 HIV-infected patients (n=66 samples) during antiretroviral therapy.

Plasma concentrations of nevirapine were 4689±2031 ng/ml. The results presented in Fig. 6 demonstrate the applicability of the assay for therapeutic drug monitoring in HIV-infected patients.

4. Conclusion

We present a validated, reliable and convenient assay for the determination of nevirapine in human plasma. The described GC assay can readily be used in a standard hospital laboratory.

In our hands, the described procedure was most suitable. Calibration curves for nevirapine from 10 to 20 000 ng/ml are appropriate for clinical drug monitoring and are especially suitable for assessment of patient's compliance.

The practicability of the assay is demonstrated by plasma levels of 12 patients regularly seen in our outpatient care unit.

This GC method is preferentially used for drug monitoring in patients treated with nevirapine in combination with other antiretroviral agents.

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