Determination of Nevirapine in Plasma by GC–MS

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Abstract

Here we describe a simple, stable, and specific gas chromatography–mass spectrometry (GC–MS) method for the determination of nevirapine in plasma. After precipitation of proteins, the non-nucleoside reverse transcriptase inhibitor nevirapine was extracted with dichloromethane. For the determination and quantification of nevirapine, 1 µL of the organic layer was injected onto the GC–MS system. Linear calibration curves were obtained with BIRH 0414BS as internal standard in a range from 0.01 to 15 µg/mL. Intra- and inter-day accuracy and precision of this method were good with an accuracy between 96–109% and a precision between 2–8% across the therapeutic range of nevirapine. GC–MS proved to be a valid alternative to high-performance liquid chromatography and liquid chromatography–MS.

Introduction

The non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine is an important antiretroviral drug used for the treatment of human immunodeficiency virus (HIV)-infection (1,2). Due to its low genetic barrier, resistance occurs rapidly if subtherapeutic drug levels occur (3). On the other hand, toxic side effects due to high nevirapine plasma levels may be observed in the case of severe liver disease compromising drug metabolism (4,5). Moreover, drug-to-drug interactions may lead to unexpected high or low nevirapine drug levels (6). Therapeutic drug monitoring of nevirapine may, thus, be helpful in individual treatment decisions by assessing the actual nevirapine plasma level (7).

Solid-phase or liquid–liquid extraction followed by high-performance liquid chromatography (HPLC) separation coupled to UV detection and quantification have been the most widely used method for the determination of nevirapine drug levels (8–13). Gas chromatography allows a faster method for detection and quantification. Moreover, erroneous peak overlapping, which may occur during UV spectral analysis, does not occur. Here we describe a simple and fast gas chromatography–mass spectroscopy (GC–MS) method for the detection and quantification of nevirapine in human plasma.

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Methods

Chemicals and reagents

Nevirapine and BIRH 0414BS (5,11-dihydro-6H-11-ethyl-4-methyl-dipyrido[3,2-b:2',3'-e]1,4]diazepin-one) were kindly provided by Boehringer Ingelheim (Ingelheim, Germany) (Figure 1). Both pharmaceuticals were certified reference compounds. The reagents used were of analytical-grade. Sodium hydroxide, dichloromethane was obtained from Merck (Darmstadt, Germany).

Standard preparation

The stock solution of BIRH 0414 BS was prepared by dissolving 5 mg BIRH 0414BS in 100 mL ethanol. 2 mL of the stock solution (= 1 mg BIRH 041 BS) was diluted with 8 mL ethanol to obtain the final working solution with a concentration of 0.1 mg/mL BIRH 0414 BS.

The stock and working solution of nevirapine was prepared in the same manner, obtaining a working solution with a concentration of 0.1 mg/mL nevirapine. Stock solutions were stored at 4°C in order to guarantee a stable solution over a time period of three months.

Sample preparation

To a 500 µL aliquot of human plasma placed in a 10-mL tube, 20 µL of the internal standard solution (0.1 mg/mL BIRH 0414 BS in ethanol) and 100 µL 0.1M sodium hydroxide was pipetted. After the addition of 500 µL of acetonitrile, the suspension was vortexed for 30 s on a laboratory vortex followed by centrifugation (10 min, 3500 g) at room temperature. The supernatant was

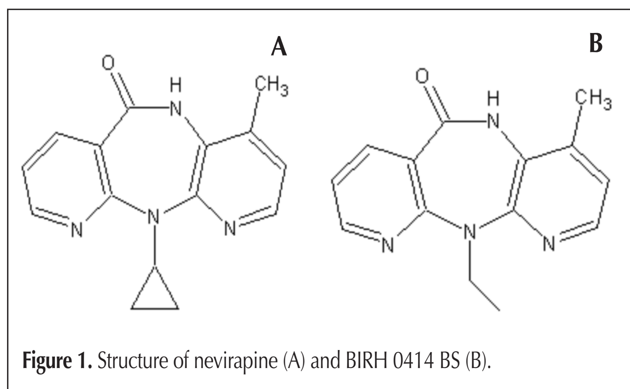

Figure 1. Structure of nevirapine (A) and BIRH 0414 BS (B).

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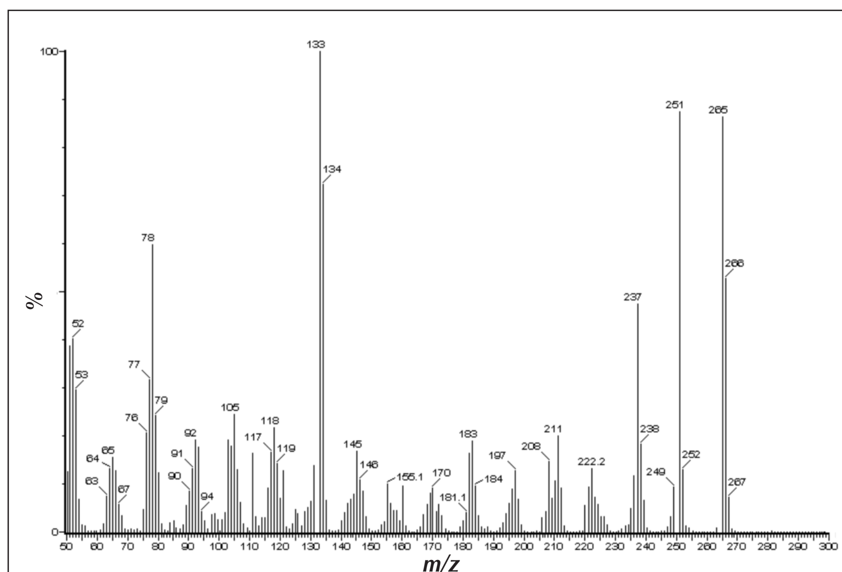

Figure 2. EI-MS-spectrum of nevirapine.

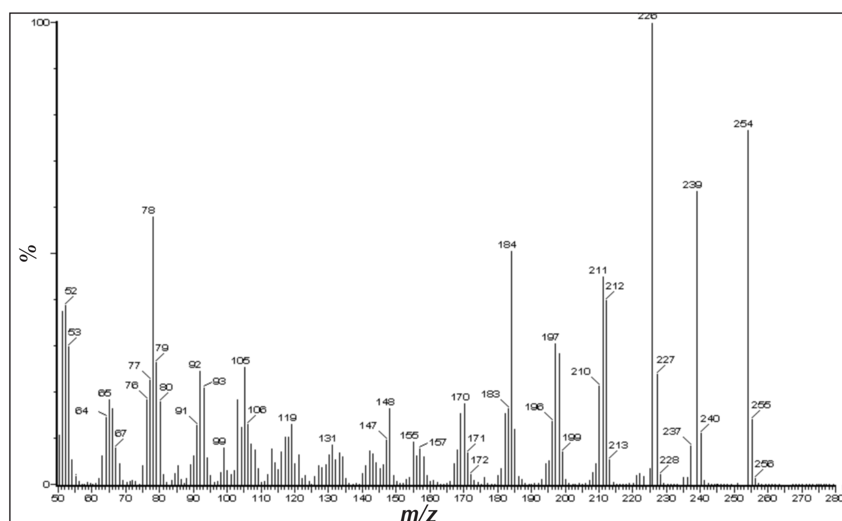

Figure 3. EI-MS-spectrum of BIRH 0414 BS.

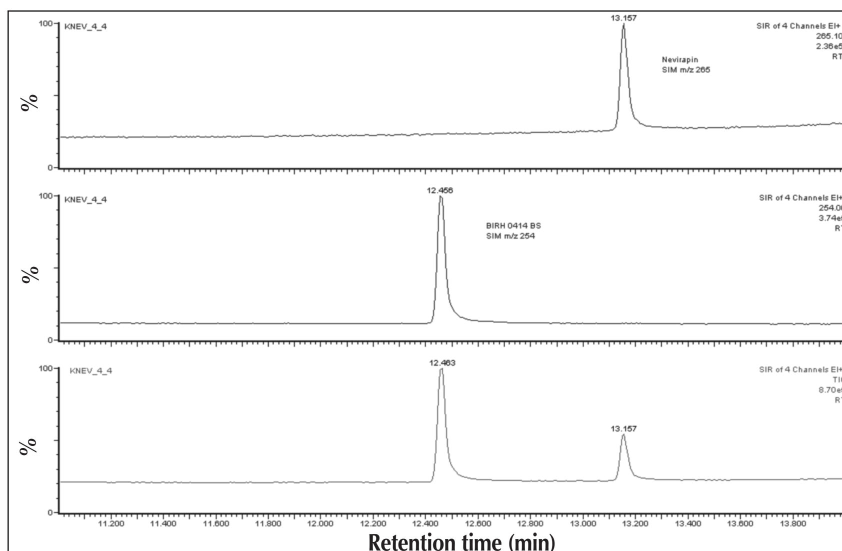

Figure 4. GC–MS total ion chromatogram of the extract from a 500-µL human plasma sample containing 4 µg/mL Nevirapine.

transferred into a second 10 mL tube, and the organic phase was extracted two times with 1 mL dichloromethane by shaking on a vortex mixer for 30 s and centrifugation (10 min, 3500 *g*) at room temperature. 100 µL of the extracted organic phase was transferred in an autosampler vial for the direct analysis of nevirapine.

Measurement

GC conditions

A Fisons GC 8065 capillary GC with split-splitless injector and A200S auto sampler, coupled to a Fisons MD800 mass spectrometer was used (Thermo Fisher Scientific, Waltham, MA). Injection was done in splitless mode (1 min). The carrier gas was helium, purity 99.999% (Messer-Griesheim, Krefeld, Germany); the inlet pressure was set to 80 kPa. The column used was an HP1 (30 m × 0.32 mm i.d., 0.25 µm film thickness) from Agilent (Santa Clara, CA). The injector temperature was 200°C; the interface temperature 250°C. The temperature program was set to 1 min isothermal 70°C, increase to 250°C with 15°/min, increase to 320°C with 25°C/min, and then another 1 min isothermal.

MS conditions

The $(M)^+$ ion from nevirapine and BIRH 0414BS was measured after 70 eV electron impact ionization: *m/z* 254 and 265. Scan time per ion was 0.2 s, dwell time per ion was 0.08 s, solvent delay was 10.0 min, ion source temperature 220°C, and trap current 100 µA. Prior to the start of each series, the MS was tuned, and one spiked sample was run with the temperature program to localize the maximal sensitivity for the analytes. The analysis series was started with parameters adjusted accordingly, if required (Figure 2 and 3).

Patients

For the obtainment of nevirapine plasma samples, one patient from the HIV-outpatient clinic at Bonn University was enrolled. To be included, the patient had to be HIV-mono-infected and on a stable nevirapine containing highly active antiretroviral therapy (HAART) for more than four weeks. Nevirapine was dosed 200 mg twice daily. Coexisting liver disease, such as chronic hepatitis B, C, or concomitant medication with potential drug-to-drug interactions had to be ruled out. After written informed consent, the patient was admitted to our day clinic for 12 h in order to perform a 12 h pharmacokinetic (PK) study. The patient had to visit the clinic at the

morning of the PK study in a fasted state. After a first blood withdrawal (trough level, 0 hour), a standard breakfast together with one tablet of 200 mg nevirapine was taken, and consecutive blood withdrawals for PK sampling were taken at 1, 2, 4, 6, and 12 h thereafter. Standard lunch and dinner meals were issued to the patient 3.5 and 8 h after intake of the morning dose of nevirapine. The study had been reviewed by the local ethics committee and was conducted in agreement with good clinical practice and the declaration of Helsinki and its subsequent revisions.

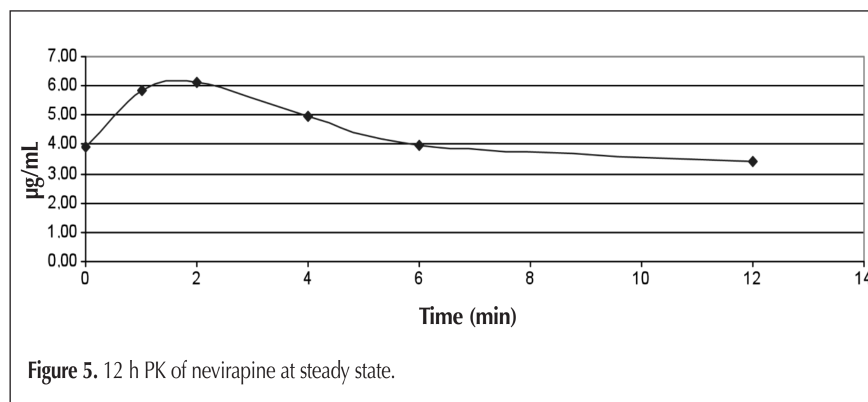

Figure 5. 12 h PK of nevirapine at steady state.

Table I. Long Term Stability of Nevirapine*

Conditions	Spiked conc (µg/mL)	Measured conc. (µg/mL) (SD)
48 h; 21°C	5.00	4.98 (0.09)
Three months (–80°C)	5.00	5.04 (0.12)

* Long term stability of nevirapine in plasma samples was tested over 48 h at 21°C and for 3 months frozen at –80°C. Spiked conc. spiked plasma concentration of nevirapine; Measured conc. measured nevirapine plasma concentration after 48 h / 3 months, standard deviation in brackets.

Table II. Inter- and Intra-Day Precision and Accuracy of Nevirapine in Human Plasma Using GC–MS

	Nominal Conc. of nevirapine (µg/mL)			
	1.00	**2.00**	**5.00**	**7.00**
Conc. found	*µg/mL, arithm. mean value(SD)*			
Day 1 (n = 6)	1.05 (0.03)	2.08 (0.05)	5.12 (0.13)	7.06 (0.18)
Day 2 (n = 6)	1.09 (0.04)	1.91 (0.09)	4.95 (0.08)	6.98 (0.12)
Day 3 (n = 6)	1.03 (0.02)	2.09 (0.06)	5.25 (0.17)	6.86 (0.15)
Inter-day (n = 18)	1.12 (0.04)	2.03 (0.06)	5.18 (0.14)	6.97 (0.18)
Accuracy	*(%, arithm. mean value)*			
Day 1 (n = 6)	105	109	102	101
Day 2 (n = 6)	109	96	99	100
Day 3 (n = 6)	103	104	105	98
Inter-day (n = 18)	105	103	102	100
Precision	*(%, arithm. mean value)*			
Day 1 (n = 6)	2.9	2.1	2.5	2.6
Day 2 (n = 6)	3.4	4.6	1.7	1.7
Day 3 (n = 6)	1.8	3.0	2.6	2.2
Inter-day (n = 18)	3.9	5.2	3.2	2.5

Results

Chromatographic separation

Nevirapine and the internal standard were completely separated under the chromatographic conditions chosen (Figure 4).

Calibration curves

The relationship between the concentration of nevirapine and the peak area ratio of nevirapine and the internal standard was analyzed. A linear relationship was obtained between the concentration of nevirapine and the peak area ratio of nevirapine and the internal standard. The calibration curves were linear, with coefficients of determination (r^2) greater than 0.999 in the clinically relevant range of 1–7 µg/mL for nevirapine.

Sensitivity

Nevirapine was quantified at m/z 265. The limit of detection was 0.001 µg/mL nevirapine in plasma. At these concentration, the signal-to-noise ratio was 5:1. The limit of quantification was 0.01 µg/mL in plasma, with a signal-to-noise ratio better then 20:1.

Extraction efficacy

Recovery of nevirapine was calculated by comparison of the peak area ratios obtained after extraction of spiked plasma samples with spiked 2 mL dichlorormethane aliquots. The recovery of nevirapine was assessed at two different concentration levels (2.0 and 4.0 µg/mL, n = 10). The arithmetic mean value for 2.0 µg/mL was 100.9% (SD 5.0%; range 91.6–108.1%) and for 4.0 µg/µL 98.2% (SD 3.2%; range 95.1–104.1%).

Stability of nevirapine

Long-term stability of nevirapine was assessed in spiked plasma samples stored frozen at –80°C for at least three months. Stability at ambient temperature was checked for a period of 48 h (Table I).

Precision and accuracy

Assay precision and accuracy were assessed by analyzing six spiked plasma samples at clinical relevant concentrations of 1, 2, 5, and 7 µg/mL nevirapine analyzed within one day (intra-day variability) and on three consecutive days (inter-day variability). Results are shown in Table II. Throughout the therapeutic range of nevirapine, good intra- and inter-day accuracy and precision of the GC–MS method were observed.

Analysis of patient samples

Practical application of the described GC–MS-method was tested by determination of a 12 h PK-study of an HIV-infected individual (Figure 5).

Discussion

The determination of nevirapine plasma levels is most frequently performed by HPLC (8–13). However, in certain settings,

it may be beneficial to use a GC–MS instead. Since the advent of HAART, AIDS related mortality has been dramatically reduced, and other non-AIDS related co-morbidities have become a more and more important aspect of care in HIV-infected patients. As a result, poly-pharmacy in HIV-infected patients has become common and, thus, peak overlapping due to UV spectral analysis may lead to false nevirapine plasma levels.

Previous groups have used MS in conjunction with liquid chromatography (LC) (14–19); however, LC takes longer and may not be readily available. Here we described a GC–MS technique for the detection of nevirapine, which proved to have good intra- and inter-day precision and accuracy, and we were able to demonstrated its use in clinical setting.

GC–MS may, thus, be an alternative technique for the determination and quantification of nevirapine in plasma.

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