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Rapid Determination of Nevirapine in Human Plasma by High- Performance Liquid Chromatography

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ABSTRACT

Objective: To develop and validate a high-performance liquid chromatography (HPLC) method for the determination of nevirapine in human plasma.

Methods: A plasma sample and an internal standard were extracted with tert-butyl methyl ether and determined nevirapine concentration by HPLC method. The limit of quantitation (LOQ), accuracy, precision, specificity, stability and recovery were tested for method validation.

Results: Standard curve was linear in the range 0.1 μg/mL to 20 μg/mL. The limit of quantitation was 0.1 μg/ml. Coefficients of variation (CV) of intra-day and inter-day precision were less than 4%. Accuracy was range from 97-101%. The extraction recovery was range from 94-112%.

Conclusion: A rapid, sensitive and specific HPLC method was developed and can be used for determination of plasma nevirapine concentration in adult and pediatric patients infected with HIV.

Keywords: HPLC; HIV; nevirapine

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Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and binds directly to the viral reverse transcriptase to block polymerase activity by causing a disruption of the enzyme's catalytic site.[1] However, the emergence of highly drug-resistant virus has been observed within 4 weeks after initiation of monotherapy with nevirapine.[2] Nevirapine contains antiretroviral combination which will be more effective than a single drug. Since the year 2000, the Thai Governmental Pharmaceutical Organization (GPO) has produced a fixed-dose combination pill of lamivudine (3TC), stavudine (d4T) and nevirapine (NVP), namely GPO-VIR. Recently, GPO-VIR has become widely used in Thailand.[3]

Nevirapine is metabolized by the cytochrome P450 (CYP) system, and may act as either inducer or inhibitor of other drugs that are also metabolized by CYP.[4] These drug interactions are an important factor to be considered in the clinical use of these agents as a part of combination antiretroviral therapy. Drug monitoring could ensure optimal drug efficacy, toxicity and prevent viral resistance.

Several HPLC methods for determination of nevirapine in plasma have been published.[5-8] These techniques performed by liquid-solid extraction or protein precipitation without internal standard, neither of which is easy for routine application. Thus, the aim of this study is to develop and validate a simple, sensitive and rapid HPLC method for the determination of nevirapine level in routine laboratory.

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MATERIALS AND METHODS

1. Chemicals

Nevirapine (NVP) was obtained from the Thai Governmental Pharmaceutical Organization. Internal standard (IS), 3-isobutyl-methylxanthine was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol and tert-butyl methyl ether were purchased from LABSCAN (Bangkok, Thailand).

2. Instruments and HPLC conditions

The HPLC system consisted of a Waters (Milford, MA, USA) Alliance liquid chromatography system, including a Model 2695 Separate Module and a Model 2487 Dual Wavelength UV detector. Reversed-phase liquid chromatography was performed at 35°C using a Luna® C18(2) analytical column, 5 μm (250 x 4.6 mm I.D.)

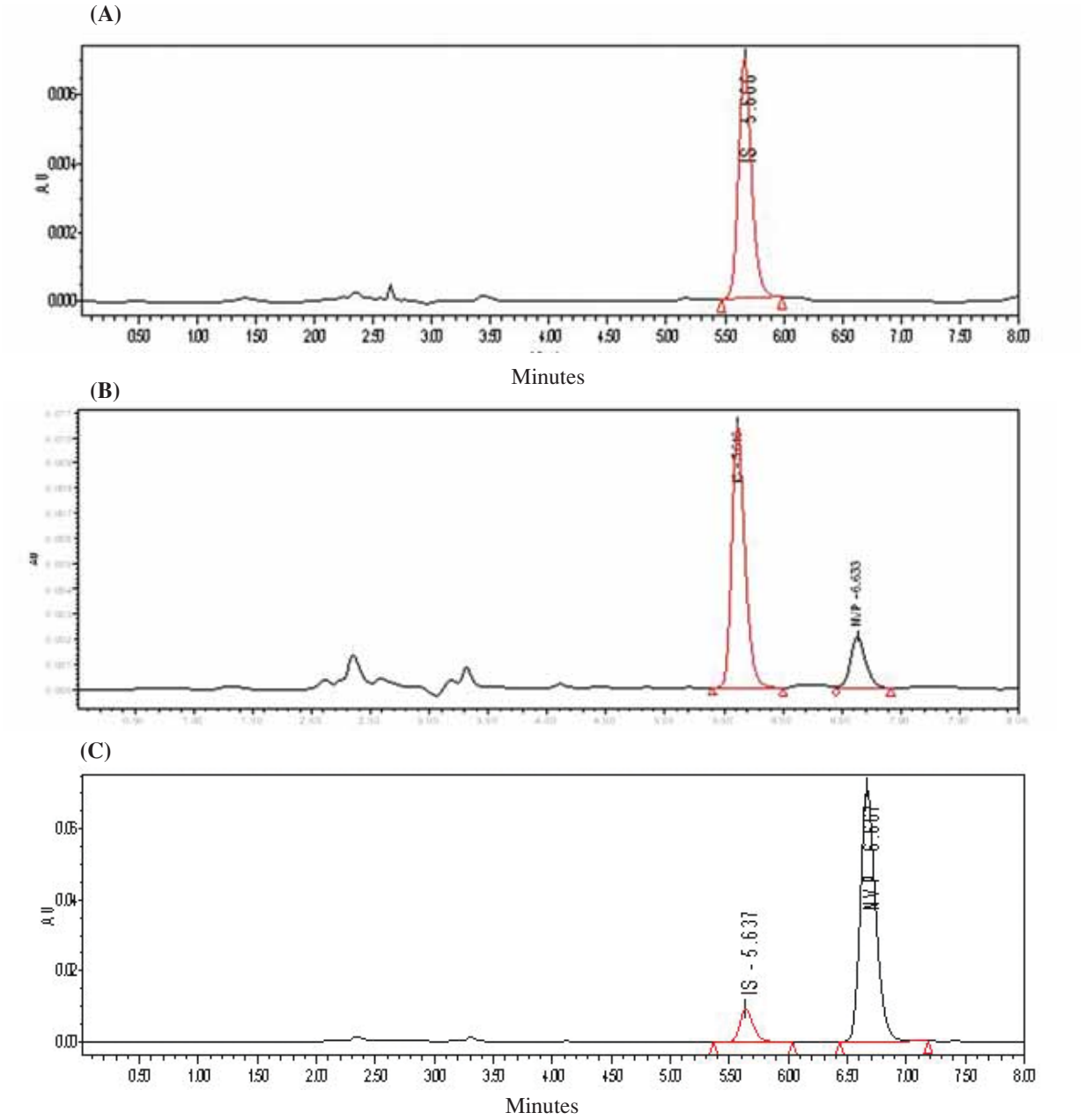


Fig 1. Chromatograms of human plasma samples (A) blank plasma; (B) blank plasma spiked with 0.1 µg/mL of nevirapine (lower limit of quantitation) and internal standard; (C) blank plasma spiked with 10 µg/mL of nevirapine and internal standard.

and protected with guard cartridge C18. The column and guard cartridge were purchased from Phenomenex (CA, USA). The isocratic mobile phase was consisted of 50 mM phosphate buffer (pH 5.6) - acetonitrile at a ratio of 70:30, v/v. The mobile phase was filtered through a 0.45 µm membrane prior to use. The UV absorbance was used at 240 nm. The flow-rate was 1 mL/min. The analysis time was set at 8 min per sample.

3. Preparation of standards

A stock solution of nevirapine was prepared in methanol at 2,000 µg/mL. For preparation of calibration standard, the stock solution was diluted in 50% methanol to a final concentration of 200 µg/mL. Calibration standard covering the concentration range between 0.1 and 15 µg/mL (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15 and 20 µg/mL) were prepared by adding appropriate volumes of these diluted solutions to drug free human plasma. The quality control (QC) samples in the concentration of 0.375, 3.75 and 15 µg/mL were prepared in the same way with calibration standard preparation. All calibration and quality control standards were divided into 250 µl aliquots and frozen at -20°C until used.

A stock solution of internal standard (IS) was prepared at 1,000 µg/mL in methanol and was diluted to 100 µg/mL in 50% methanol and kept at -20°C until used.

4. Sample preparation

A 100 µl of internal standard was added to each tube of 200 µl of plasma (patient sample, calibration and QC standard). Then 1 ml of tert-butyl methyl ether was added to each tube and the tubes were vortex mixed for 10 min. The organic phase was separated by centrifugation at 15,000 rpm for 5 min at 4°C. Afterwards the upper organic phase was evaporated to dryness under gentle stream of nitrogen at room temperature. The residue was resuspended in 250 µL of mobile phase by vortexing for 2 min and 10 µL of the solution was injected onto HPLC system.

TABLE 1. Intra-day and inter-day precision and accuracy of nevirapine in human plasma.

Expected concentration (μg/ml)	Calculated concentration (mean±SD)	CV (%)	Accuracy (%)
Intra-day (n = 6)			
0.375	0.377±0.007	1.8	101
3.75	3.641±0.030	0.8	97
15	14.932±0.057	0.4	99
Inter-day (n = 18)			
0.375	0.372±0.015	3.95	99
3.75	3.666±0.119	3.25	98
15	14.966±0.284	1.90	99

TABLE 2. Stability of nevirapine in human plasma.

Conditions	Concentration (μg/ml)	Recovery (%)	CV (%)
Heat inactivation (n=3)			
	0.375	96.98	8.0
	15	97.35	1.6
Storage at room temperature (n=3)			
	0.375	100.35	5.6
	15	99.50	2.2
Three freeze-thaw cycles (n=3)			
	0.375	99.73	0.7
	15	99.52	0.1

5. Extraction recovery

Recovery was determined by comparing the amount of nevirapine from extracted standard sample with non-extracted standard sample at the same concentration in 3 separate runs.

6. Accuracy and precision

Accuracy, intra-day and inter-day precision were determined by analyzing 6 replicate QC samples at three different concentrations (0.375, 3.75 and 15 μg/mL) for 3 separate days.

7. Selectivity

Selectivity was determined by comparing the chromatogram of spiked plasma with drug free plasma.

8. Stability

Stability testing was determined by analyzing QC samples under various conditions. The QC samples at low (0.375 μg/mL) and high (15 μg/mL) concentrations of nevirapine were separated into 3 sets. The first set was heated at 58°C for 30 min to inactivate the HIV. The second set was subjected to 3 freeze-thaw cycles and the third set was stored at room temperature for 24 h. Each QC samples were analyzed by comparing with same concentration of freshly thawed QC samples.

9. Calibration and statistical analysis

In this study, EmpowerPro software (Water, Milford, MA, USA) was used to generate the calibration curve by plotting the areas under curve ratio of nevirapine / IS of extracted spike plasma versus various concentrations of nevirapine. The values from the linear regression were used for calculation of nevirapine concentrations in the samples from their areas under curve ratio.

RESULTS

The chromatogram of drug-free plasma and spiked plasma with internal standard are shown in Fig 1. There were no interfering peaks in drug-free plasma at the retention time of nevirapine and internal standard. The mean retention time of internal standard and nevirapine were 5.725 and 6.791 min, respectively. The assay run time was only 8 min.

A least-square linear regression was used to calculate the equation relating the peak-area ratio between drug/IS and the concentration of nevirapine. The calibration curve was linear in the range of 0.1 μg/mL to 20 μg/mL with correlation coefficient higher than 0.99 (CV < 10%). The lower limit of quantitation (LOQ) for this assay was 0.10 μg/mL.

The results obtained for precision and accuracy are shown in Table 1. The intra-day and inter-day coefficients of variation (CV) of nevirapine ranged from 0.4 to 1.8% and 1.9 to 4.0%, respectively. Accuracy ranged from 97 to 101%.

Recovery was calculated by comparing the peak area after extraction from plasma with the peak area after injection of the same concentration of nevirapine dissolved in mobile phase. The mean recovery of nevirapine (n = 3) after extraction were 94.7, 96.8, 95.5, 93.2, 96.5, 95.2, 104.7, 99.4 and 111.5% for nevirapine concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20 μg/mL, respectively. The CV values range from 0.32 to 10.96%.

The stability of nevirapine under various conditions at two concentrations of QC standards are shown in Table 2. Nevirapine was stable for 24 h at room temperature and for heated at 58°C for 30 min. No degradation was observed at -20°C after three repeated freeze-thaw cycles. At least 96% of the initial concentrations were recovered. These results are in agreement with those reported in the literature.[5-8]

DISCUSSION

A simple, rapid and sensitivity HPLC assay was developed and validated for determination of nevirapine in human plasma. This method requires a small plasma volume (200 μL) which allows the analysis of pediatric sample where blood volumes are limited. Despite a lower plasma volume, the LOQ in this study is similar to previous reports.[5-8]

CONCLUSION

In conclusion, this assay has been completely validated with respect to precision, accuracy, stability, LOQ, recovery and linearity. It can be used for monitoring of plasma nevirapine level in HIV-infected patients with potential drug-drug interactions or for whom optimal nevirapine concentration is critical for effective treatment.

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บทคัดย่อ

การตรวจหาปริมาณ nevirapine ในกระแสเลือดด้วยวิธีโครมาโทกราฟีของเหลวแบบสมรรถนะสูงที่ได้ผลรวดเร็ว

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วัตถุประสงค์: เพื่อพัฒนา และตรวจสอบความถูกต้องของวิธีการวิเคราะห์ความเข้มข้นของยา nevirapine ในพลาสมา ด้วยวิธีโครมาโทกราฟีของเหลวแบบสมรรถนะสูง (high-performance liquid chromatography ; HPLC)

วิธีการ: สกัดยา nevirapine ออกจากพลาสมา โดยใช้ tert-butyl methyl ether และวิเคราะห์หาความเข้มข้นของยา nevirapine ด้วยวิธี HPLC การตรวจสอบความถูกต้องของวิธี HPLC ที่พัฒนาขึ้น ประกอบด้วยการตรวจหาระดับความเข้มข้นที่น้อยที่สุดที่สามารถวัดปริมาณได้ (limit of quantitation), การตรวจสอบความถูกต้อง (accuracy), ความแม่นยำ (precision), ความจำเพาะ (specificity), และ การตรวจสอบความสามารถของวิธีสกัดยา (recovery) เป็นต้น

ผลการศึกษา: วิธี HPLC สามารถวิเคราะห์ปริมาณยา nevirapine ในระดับต่ำสุดถึง 0.1 μg/ml คือ ได้ตั้งแต่ 0.1 μg/ml จนถึง 20 μg/ml ความถูกต้องมาวิธีการทดสอบอยู่ที่ 97-101%, ความแม่นยำของการวิธีการทดสอบมีค่าสัมประสิทธิ์การแปรผัน (CV) น้อยกว่า 4% และความสามารถของวิธีสกัดยามีค่าอยู่ระหว่าง 94-112%

สรุป: ได้วิธี HPLC ที่มีความไวสูง และให้ผลการตรวจที่ถูกต้อง แม่นยำ สำหรับการวิเคราะห์หาความเข้มข้นของยา nevirapine ที่อยู่ใน plasma ของผู้ป่วยที่ติดเชื้อเอชไอวี