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Short communication

A simple and rapid liquid chromatography method for simultaneous determination of zidovudine and nevirapine in plasma

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Abstract

We describe a simple, fast, isocratic, reversed-phase high performance liquid chromatographic method for simultaneous determination of plasma zidovudine and nevirapine with UV detection at 260 nm. The method involves liquid–liquid extraction with ethyl acetate and using 3-isobutyl 1-methyl xanthine as internal standard. The system requires a C_{18} column (150 mm × 4.6 mm I.D.) and a mobile phase composed of potassium dihydrogen phosphate (15 mM; pH 7.5) and acetonitrile in the ratio of 80:20 (v/v). The assay was linear from 0.025 to 10.0 μg/ml for zidovudine and 0.05 to 10.0 μg/ml for nevirapine. The intra- and inter-day variations were less than 10% for both the drugs. The method was specific and sensitive enough to allow quantification of zidovudine and nevirapine in concentrations observed clinically. The average recoveries of zidovudine and nevirapine from plasma were 95 and 94%, respectively. The method was applied to a pharmacokinetic study in HIV-infected patients who were receiving antiretroviral treatment with zidovudine and nevirapine containing regimens. The method spans the blood concentration range of clinical interest. Due to its simplicity, the assay can be used for pharmacokinetic studies and therapeutic drug monitoring in patients taking a combination treatment of zidovudine and nevirapine.

Keywords: Zidovudine; Nevirapine; Plasma; HPLC

1. Introduction

The current standard of care for the treatment of HIV-1 infection is triple-drug therapy with a two-nucleoside or nucleotide reverse transcriptase inhibitor backbone in combination with a non-nucleoside reverse transcriptase inhibitor or protease inhibitor [1,2]. Fixed-dose combination pills consisting of nevirapine and lamivudine with either zidovudine or stavudine are commonly used by patients in the developing countries [3].

Concurrent therapy of tuberculosis-HIV co-infection requires concomitant administration of at least two to four anti-tuberculosis agents and at least three antiretroviral drugs. Sub-therapeutic nevirapine levels due to pharmacokinetic interactions with rifampicin could lead to development of resistance and subsequent treatment failure. There is no consensus on whether therapeutic drug monitoring should be performed for nucleoside reverse transcriptase inhibitors. However, relationships between plasma concentrations and virologic efficacy or toxicity have been described for zidovudine [4]. In general, treatment success can be evaluated by a reduction of viral load and by an increase of the CD4 cell count, and to achieve optimal drug concentrations for viral suppression and avoidance of drug toxicity, monitoring of drug levels has been considered.

In the literature, numerous methods to individually quantify nevirapine [5–7] and zidovudine [8–12] have been described. Few methods to estimate nevirapine and zidovudine simultaneously have been reported [13–17]. Some of these methods have used solid phase for extraction of the drugs from plasma [15–17]. This could increase the time and cost of the assay, which may not be affordable in developing countries. Sample pretreatment by liquid–liquid extraction has been reported by Moyer et al. [13] and Donnerer et al. [14]. The method of Moyer et al. [13] has used a mixture of chloroform and isopropanol (6 ml) to extract nevirapine and zidovudine from plasma, which could take a long time for evaporation. The retention times reported by them for zidovudine and nevirapine are 4.4 and 11.2 min, respectively. Although several drugs have been tested in their interference studies, certain first-line anti-tuberculosis drugs

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such as rifampicin, ethambutol, streptomycin and second-line drug, ofloxacin have not been studied. It is important that interference due to all these anti-tuberculosis drugs are also checked, so that the method can be used in pharmacokinetic and therapeutic drug monitoring studies involving HIV-TB co-infected patients. Liquid–liquid extraction with ethyl acetate for simultaneous estimation of nevirapine and zidovudine in plasma has not been reported. We have developed a simple and rapid method that involves extraction with ethyl acetate and uses UV detection. The advantages of the present method over the published methods are the smaller sample volume required, shorter run time and simple extraction procedure.

Our aim was to develop and validate a simple and rapid HPLC assay for measuring zidovudine and nevirapine levels in plasma, which is free of interference from certain antiretroviral and anti-tuberculosis drugs and other co-medications that are commonly used to treat HIV/AIDS patients. After validation, the method was evaluated in samples collected from HIV-infected individuals who were receiving antiretroviral treatment with a fixed-dose combination pill consisting of nevirapine (200 mg), zidovudine (300 mg) and lamivudine (150 mg) twice daily.

2. Experimental

2.1. Chemicals

Pure zidovudine and nevirapine powder were kind gifts from Aurobindo Pharma, Hyderabad, India. 3-Isobutyl 1-methyl xanthine was obtained from Sigma Chemical Company, St. Louis, MO, USA. Acetonitrile (HPLC grade) was from Merck (India) and potassium dihydrogen orthophosphate was from Qualigens (India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma was obtained from Lions Blood Bank, Chennai, India.

2.2. Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), diode array detector (SPD-M10Avp) and system controller (SCL-10Avp). A rheodyne manual injector (Rheodyne, Cotati, CA, USA) attached with a 20 μl sample loop was used for loading the sample. ClassVP-LC workstation was used for data collection and acquisition. The analytical column was a C_{18}, 150 mm × 4.6 mm I.D., 5 μ particle size protected by a compatible guard column (Lichrospher 100 RP-18e, Merck, Germany).

The mobile phase consisted of 15 mM phosphate buffer pH 7.5 (adjusted with 1N NaOH) and acetonitrile (80:20, v/v). Prior to preparation of the mobile phase, the phosphate buffer and acetonitrile were degassed separately using a Millipore vacuum pump. The UV detector was set at 260 nm. The chromatogram was run for 10 min at a flow rate of 1.5 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) versus concentration curve. The linearity was verified using estimates of correlation coefficient (r).

2.3. Preparation of standard solution

Stock standards (1 mg/ml) of zidovudine and nevirapine were prepared separately by dissolving the drugs in methanol. The working standards of zidovudine (0.025 to 10.0 μg/ml) and nevirapine (0.05 to 10.0 μg/ml) in combination were prepared in human plasma. Thus each working standard solution in human plasma contained 0.05, 0.10, 0.50, 1.0, 2.5, 5.0 and 10.0 μg/ml each of zidovudine and nevirapine. An additional concentration of 0.025 μg/ml of zidovudine, which did not contain nevirapine, was also prepared.

2.4. Sample preparation

To 250 μl each of calibration standards and test samples, 25 μl of 3-isobutyl 1-methyl xanthine (internal standard) was added at a concentration of 100 μg/ml. One milliliter of ethyl acetate was added and the contents were vortexed vigorously, centrifuged at 1000 × g and 500 μl of organic phase was evaporated to dryness. The dried residue was reconstituted in 50 μl of mobile phase and 20 μl was injected into the HPLC column.

2.5. Accuracy and linearity

The accuracy and linearity of zidovudine and nevirapine standards were evaluated by analysing a set of standards ranging from 0.025 to 10.0 μg/ml for zidovudine and 0.05 to 10.0 μg/ml for nevirapine. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

2.6. Precision

In order to evaluate the precision of the method, six different concentrations of zidovudine (0.15, 0.50, 1.20, 1.75, 2.5 and 3.5 μg/ml) and nevirapine (0.45, 2.0, 2.75, 7.50, 9.0 and 12.5 μg/ml) were prepared in human plasma and analysed in duplicate on three consecutive days.

2.7. Recovery

Varying concentrations of zidovudine and nevirapine (0.1, 0.5, 1.0, 5.0, 10.0 μg/ml) together were prepared in drug-free human plasma and extracted as described above along with the internal standard. The percentage of the drug recovered from the plasma samples was determined by comparing the peak height ratio after extraction with those of unextracted methanolic solutions containing same concentrations of zidovudine and nevirapine as in plasma. Recovery experiments were carried out on three consecutive days.

2.8. Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples obtained from six each of male and female subjects. Interference from certain antiretroviral drugs, namely, efavirenz, didanosine, stavudine, lamivu-

dine, indinavir and nelfinavir, anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin and other commonly co-administered medications such as ofloxacin, acetozolamide, loperamide, prednisolone, diphenyl hydantoin, amitriptyline, cotrimoxazole and fluconazole at a high concentration of 50 μg/ml was also evaluated.

2.9. Samples

Six adult HIV seropositive individuals (3 males and 3 females) aged 30–36 years and body weight ranging from 34 to 57 kg, who were attending the out-patient clinic of the centre, took part in the study. They were on regular antiretroviral treatment consisting of nevirapine (200 mg), zidovudine (300 mg) and lamivudine (150 mg) twice daily for a period of 1–9 months. None of the patients were suffering from diabetes or severe hepatic or renal dysfunction (serum transaminases, urea and creatinine within two and a half times the upper normal limit). Chronic alcoholics and females on hormonal birth control pills were not included. On the study day, the patients were instructed to report to the clinic in a fasting condition. Blood samples (3 ml) were drawn in heparinized containers before dosing and at 0.5, 1, 2, 4, 6, 8 and 12 h after dosing. All the blood samples were centrifuged immediately and plasma was separated and stored at −20 °C until assay. Estimations of plasma zidovudine and nevirapine in all the samples were undertaken within 24–48 h of blood collection. Informed written consent was obtained from all the patients before blood draws were made.

3. Results and discussion

Methods to estimate zidovudine and nevirapine simultaneously could be advantageous for pharmacokinetic studies and therapeutic drug monitoring in patients who are receiving a combination of these drugs. In this study, we have standardised and validated a simple method that permits simultaneous determination of zidovudine and nevirapine in human plasma.

Several HPLC methods for simultaneous determination of zidovudine and nevirapine are available [13–17]. The method described here has several advantages over the published methods. The volume of plasma required is 250 μl and the volume of organic solvent used in this method, namely, ethyl acetate is only 1 ml which are lower than other methods [13,14], and this considerably reduces the sample processing time. Also, the retention times of zidovudine and nevirapine were 2.3 and 5.5 min, respectively, and this reduces the run time. Hence the present method is rapid, and extraction using ethyl acetate is simple without any loss of analytes. The use of internal standard helped in monitoring the recovery of zidovudine and nevirapine from plasma.

Under the chromatographic conditions described above, zidovudine and nevirapine were well separated as seen in the representative chromatograms (Fig. 1b and c). The retention times of zidovudine, internal standard and nevirapine were 2.3, 4.1 and 5.5 min, respectively. Blank plasma samples did not give any peak at the retention times of zidovudine and nevirapine (Fig. 1a). The lowest concentrations of zidovudine and nevirapine gave discrete peaks at 2.3 and 5.5 min, respectively (Fig. 1b).

The concomitant therapeutic agents most likely to be encountered in the plasma of HIV-positive patients were screened under the HPLC assay conditions. No endogenous substances or antiretroviral drugs such as efavirenz, didanosine, stavudine, lamivudine, indinavir and nelfinavir, anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin and other commonly co-administered medications such as ofloxacin, acetozolamide, loperamide, prednisolone, diphenyl hydantoin, amitriptyline, cotrimoxazole and fluconazole interfered with the chromatogram of zidovudine and nevirapine.

Tuberculosis is an entry point for a significant proportion of HIV-infected patients eligible for antiretroviral treatment in resource-limited settings. HIV-tuberculosis co-infected patients would be receiving treatment for both infections. It is, therefore essential that methods developed for estimation of antiretroviral drugs rule out interference of key anti-tuberculosis drugs. Most of the published methods have not included some of the first-line anti-tuberculosis drugs in their interference studies. In the present method, none of the anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin interfered in the estimation of plasma zidovudine and nevirapine. Fig. 1c represents the chromatogram of a plasma sample collected from a HIV-infected patient, who was being treated with zidovudine and nevirapine containing antiretroviral regimens in addition to fluconazole for fungal infection and prophylaxis with cotrimoxazole. Distinct peaks at 2.4 and 6.0 min for zidovudine and nevirapine, respectively correspond to 1.19 and 10.9 μg/ml concentrations of plasma zidovudine and nevirapine.

The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma zidovudine and nevirapine were tested on six consecutive days. The plasma zidovudine standard concentrations ranged from 0.025 to 10.0 μg/ml and that for nevirapine from 0.05 to 10.0 μg/ml. A linear relationship was observed between peak height ratios and concentrations over these ranges with a mean correlation coefficient of 0.9997 for zidovudine and nevirapine. Slope and intercept values for each run were calculated using the equation $y = a + bx$, where a is intercept, b the slope, x the standard concentration and y is peak height ratio. The mean (± SD) slope and intercept values for zidovudine were 0.291 ± 0.006 and -0.00441 ± 0.000537, respectively. The corresponding values for nevirapine were 0.137 ± 0.005 and 0.0062 ± 0.0031. The within-day and between-day relative standard deviation (RSD) for zidovudine standards ranged from 3.4 to 5.9% and 1.8 to 9.9%, respectively. The corresponding RSD ranges for nevirapine were 3.0 to 9.0% and 2.9 to 8.9%.

The reproducibility of the method was further evaluated by analysing six plasma samples containing different concentrations of zidovudine and nevirapine. The RSD for these samples ranged from 2.7 to 6.8% for zidovudine and 2.7 to 8.5% for nevirapine (Table 1). The percent variations from the actual concentrations ranged from 96 to 107% for zidovudine and 89 to 102% for nevirapine. For the concentration to be accepted as the lowest limit of quantification (LOQ), the RSD (measure of precision) has to be less than 20% [15]. In the present method,

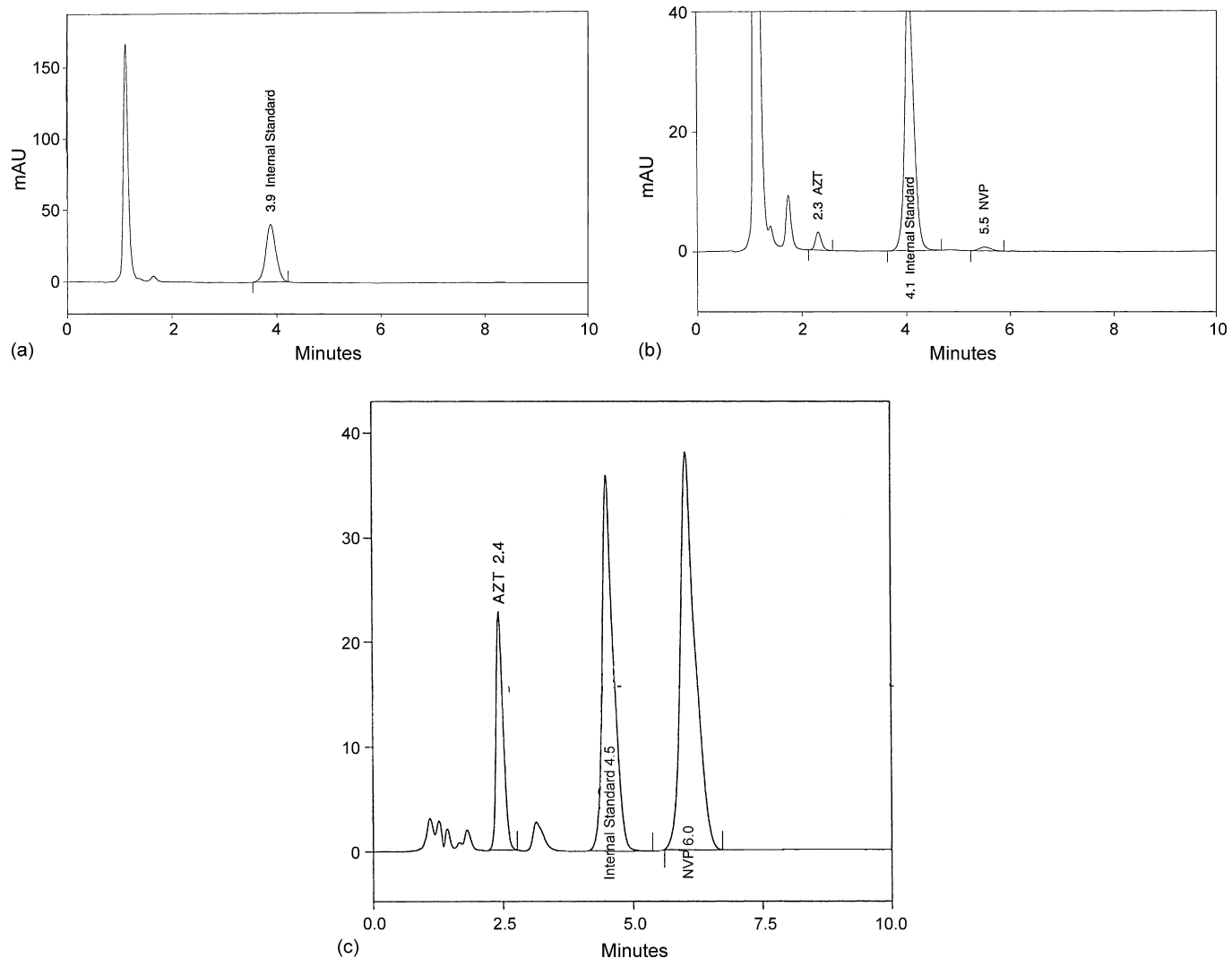

Fig. 1. (a) Chromatogram of extracted blank plasma; (b) chromatogram of extracted zidovudine (0.025 μg/ml) and nevirapine (0.05 μg/ml) plasma standards; (c) chromatogram of extracted plasma sample of HIV-infected patient.

the lowest concentrations of zidovudine and nevirapine in the calibration curve were 0.025 and 0.05 μg/ml, respectively. The within-day and between-day RSD values for these concentrations were less than 10%. Hence the LOQ for zidovudine and nevirapine can be taken as 0.025 and 0.05 μg/ml, respectively. The absolute recovery of zidovudine and nevirapine from plasma extracted with ethyl acetate was calculated by comparing the peak height ratio of five concentrations of zidovudine and nevirapine in human plasma that underwent extraction to that of identical concentrations of zidovudine and nevirapine prepared in methanol without extraction. This method reliably eliminated interfering material from plasma, yielding recoveries for zidovudine and nevirapine that ranged from 92 to 103% and 86 to 102%, respectively.

Table 1
Precision of plasma zidovudine and nevirapine assay

Sample no.	Zidovudine		Nevirapine	
	Actual concentration (μg/ml)	Found concentration (μg/ml) mean ± SD (% RSD)	Actual concentration (μg/ml)	Found concentration (μg/ml) mean ± SD (% RSD)
I	0.15	0.16 ± 0.01 (5.9)	0.45	0.44 ± 0.01 (2.7)
II	0.50	0.49 ± 0.03 (5.4)	2.0	1.77 ± 0.08 (4.8)
III	1.20	1.17 ± 0.08 (6.8)	2.75	2.63 ± 0.20 (7.4)
IV	1.75	1.68 ± 0.04 (2.7)	7.5	7.28 ± 0.51 (7.0)
V	2.5	2.43 ± 0.09 (3.8)	9.0	9.20 ± 0.78 (8.5)
VI	3.5	3.53 ± 0.23 (6.5)	12.5	12.28 ± 0.92 (7.5)

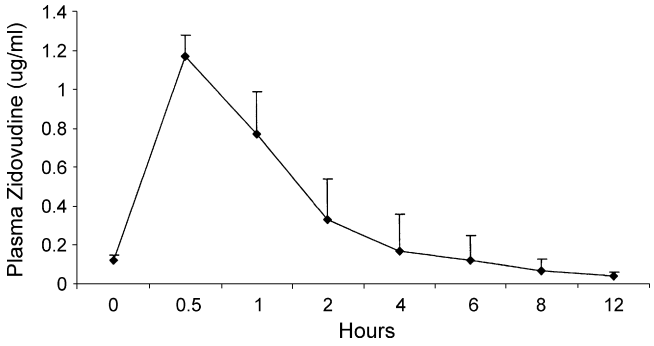

Fig. 2. Mean steady state plasma zidovudine concentrations of HIV-infected patients ($n = 6$) at different time points (vertical bars represent SD).

The method described was applied for the determination of zidovudine and nevirapine concentrations in blood collected from six HIV-infected individuals who were undergoing treatment with zidovudine and nevirapine-containing regimens. The mean plasma zidovudine and nevirapine concentrations at steady state during a dosing interval (0–12 h) are shown in Figs. 2 and 3, respectively. The mean peak and trough concentrations of zidovudine observed in this study were 1.17 and 0.04 μg/ml. The time to attain peak concentration was 0.5 h. While these values are similar to those reported by some [18,19], they differ from those reported by others [14,20]. In the case of nevirapine, peak and trough concentrations obtained in this study were 8.80 and 5.81 μg/ml, respectively, which are higher than those reported earlier [14,20–24]. The only study that reported higher peak (9.3 μg/ml) and trough (7.65 μg/ml) concentrations than the present study data was that done in an African population [25]. Genetic differences among populations could lead to differences in antiretroviral drug concentrations, toxicity and outcome. A CYP2B6 allelic variant (G516T) has been associated with greater plasma exposure to nevirapine [26]. Higher blood concentrations of zidovudine and nevirapine observed in this study are from a small number of patients. It would therefore be important to study the pharmacokinetics of antiretroviral drugs in Indian patients.

In conclusion, a sensitive, specific and validated assay for the simultaneous quantitative determination of zidovudine and nevirapine in plasma is described. The method is rapid and utilizes a single step extraction. The chromatogram yields well-resolved peaks for zidovudine and nevirapine with good intra- and inter-day precisions. The easy sample preparation and small sample volume makes this method highly suitable for pharmacokinetic studies and therapeutic drug monitoring in patients with HIV and HIV-tuberculosis on antiretroviral therapy.

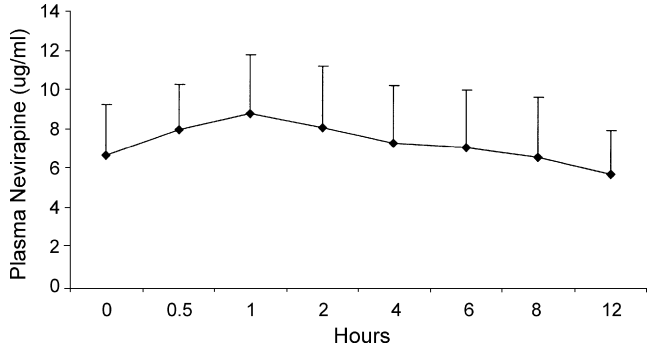

Fig. 3. Mean steady state plasma nevirapine concentrations of HIV-infected patients ($n = 6$) at different time points (vertical bars represent SD).

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