

## Research Article

# Simultaneous quantitative determination of zidovudine and nevirapine in human plasma using isocratic, reverse phase high performance liquid chromatography

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## Abstract

**Purpose:** To develop a sensitive and rapid reverse phase high performance liquid chromatography (HPLC) method for the measurement of the levels of zidovudine (ZVD) and nevirapine (NVP) in human plasma.

**Methods:** Standard stock solutions for HPLC analysis were prepared by dissolving ZVD and NVP in methanol. In the HPLC measurement, sample detection was carried out at 246 nm using an ultraviolet (UV)-photo diode array (PDA) detector. Plasma sample pretreatment consisted of protein precipitation extraction with methanol. The compounds were separated using a mobile phase consisting of a pH 3.0 solution (obtained by adjusting the pH of water with orthophosphoric acid): acetonitrile (73:27 v/v) on a Phenomenex LUNA C<sub>18</sub>, column (250×4.6 mm i.d., 5µm) at a flow rate of 0.9 mL min<sup>-1</sup>. The total run time for the assay was 10.2 min. The method was validated over the range of 300-9600 ng mL<sup>-1</sup> and 200-6400 ng mL<sup>-1</sup> for ZVD and NVP, respectively.

**Results:** The lowest limits of quantification (LLOQ) and of detection (LOD) were 300 and 63 ng mL<sup>-1</sup> for ZVD and 200 and 17 ng mL<sup>-1</sup> for NVP, respectively. The method was found to be accurate, with accuracy ranging from -10.92 to +9.57 % and precise, with intra-day, inter-day as well as analyst to analyst precision of 0.68 to 9.38 %. Extraction recoveries of the drugs from plasma were 91.39, 95.01, 89.51 % for ZVD and 90.93, 93.26, 92.13 % for NVP, for LQC (low quality control), MQC (medium quality control) and HQC (high quality control) samples, respectively. Stability data revealed that the drugs were stable in plasma under various test conditions.

**Conclusion:** This assay can be suitably used for the determination of zidovudine (ZVD) and nevirapine (NVP) in human plasma and should be useful in HIV clinical trials and clinical therapeutic drug monitoring (TDM) programs. It would also be potentially useful in the determination of pharmacokinetic profiles and in bioequivalence studies in HIV research.

**Keywords:** Assay, Zidovudine, Nevirapine, Human plasma, Reverse phase high-performance liquid chromatography.

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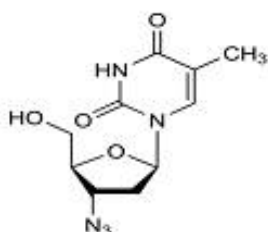
## INTRODUCTION

Major advances have been accomplished in recent years in the treatment of patients infected with human immunodeficiency virus-type 1 (HIV-1). However, many patients experience treatment failure within one year after initiation of antiretroviral therapy<sup>1</sup>. Combination therapy has now become the standard line of treatment to manage acquired immunodeficiency syndrome (AIDS)<sup>2</sup>. The need for such a therapy has arisen due to the development of resistance by human immunodeficiency virus (HIV), to single anti-HIV drugs and also in order to minimize the dose-dependent side effects produced by these drugs<sup>3</sup>. Many drugs have been tested for their activity against HIV-1. Nevirapine (NVP), a representative of a new class of antiretroviral drugs - the non-nucleoside reverse transcriptase inhibitors (NNRTIs) - i.e., 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido- [3,2-b:2,3-e] [1,4] diazepin-6-one, and zidovudine (ZVD), i.e., 3-azido- 3-deoxythymidine, are synthetic nucleoside analogues used for the treatment of HIV infections (Fig. 1).

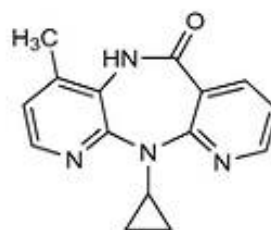
Several multi-drug high-performance liquid chromatography (HPLC) assays that measure a number of NNRTIs have been described in

the literature in recent years. Numerous analytical methods, such as HPLC with UV detection, mass spectrometry detection and immunoassay, have been reported for ZVD and NVP but they either use a gradient method for separation or a complicated liquid-liquid extraction method for preparation of plasma samples and also often involve complicated chromatography techniques. In the literature, numerous methods to individually quantify zidovudine<sup>4-7</sup> and nevirapine<sup>8-11</sup> have been described. A few methods to estimate zidovudine and nevirapine simultaneously have also been reported<sup>12-17</sup>. Some of these methods used liquid-liquid<sup>12-14</sup> and solid phase extraction procedure for the extraction of the drugs from plasma<sup>15-17</sup>. This could increase the time and cost of the assay. However, a combination of several characteristics makes this assay a unique and a useful analytical tool for quantification of these drugs.

The objective of this work was to devise a simultaneous technique for the quantitative evaluation of zidovudine (ZVD) and nevirapine (NVP) in human plasma which involves rapid protein precipitation extraction and based on simple chromatographic conditions with detection at a single wavelength (246 nm) over a short run time.



Zidovudine (ZVD)



Nevirapine (NVP)

**Fig. 1:** The chemical structure of drugs

## EXPERIMENTAL

### Reagents and chemicals

Zidovudine and nevirapine were kindly provided by Ranbaxy Laboratories Ltd, Dewas, (M.P.), India. Methanol and acetonitrile were of HPLC grade and purchased from Merck Ltd, New Delhi, India. Water used was of HPLC grade water (Mili Q Water) was purchased from Ranbaxy Laboratories Ltd, (New Delhi, India). Human plasma was obtained from Blood Bank Department, Bhopal Memorial Hospital & Research Centre, Bhopal, (M.P.), India. All other chemicals were of analytical grade and used without further purification.

### Instrumentation

The HPLC system (Shimadzu, Japan) consisted of a LC-10AT VP pump, a SPD-10A VP, PDA detector, a Phenomenex, Luna C18 (250mmX4.6mm, 5 $\mu$ m) column, a Phenomenex, HPLC guard cartridge system and a Class LC10/M10A software.

### Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a Phenomenex LUNA analytical column with a mobile phase composed of water (pH 3.0, adjusted with orthophosphoric acid): acetonitrile (73:27, v/v) and was isocratically eluted at a flow rate of 0.9 mL min<sup>-1</sup>. A small sample volume of 20  $\mu$ L was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 246 nm.

### Preparation of the calibration standards, mixed standard and quality control (QC) samples

Standard stock solutions were prepared by dissolving 60 mg of ZVD and 40 mg of NVP in 100 mL of methanol to obtain final concentrations of 600 and 400  $\mu$ g mL<sup>-1</sup> for ZDV and NVP, respectively (stock-A). From

this stock, 1 mL was taken and diluted to 10 mL with methanol to give 60  $\mu$ g mL<sup>-1</sup> of ZVD and 40  $\mu$ g mL<sup>-1</sup> of NVP standard solutions, respectively (Stock-B). Aliquots of stock-B were further diluted to obtain different concentrations: 300, 600, 1800, 3000, 4800, 6000, 7800, 9600 ng mL<sup>-1</sup> for ZDV and 200, 400, 1200, 2000, 3200, 4000, 5200, 6400 ng mL<sup>-1</sup> for NVP. To prepare standard spike plasma stock solutions, 1 mL was taken from stock-A, and diluted to 10mL with blank plasma to give 60  $\mu$ g mL<sup>-1</sup> of ZVD and 40  $\mu$ g mL<sup>-1</sup> of NVP (stock-C). From the working standard solution stock-C, different concentrations were prepared by taking 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 1.8 mL, and then the volume was made up, in each case, to 2 mL with blank plasma. For all the concentrations, 8 mL of precipitating agent (methanol) was added to obtain the final concentrations of 300, 600, 1800, 3000, 4800, 6000, 7800, 9600 ng mL<sup>-1</sup> for ZVD and 200, 400, 1200, 2000, 3200, 4000, 5200, 6400 ng mL<sup>-1</sup> for NVP.

QC samples at three different levels of HQC (high quality control, 6000/4000 ng mL<sup>-1</sup>), MQC (medium quality control, 3000/2000 ng mL<sup>-1</sup>), LQC (low quality control, 600/400 ng mL<sup>-1</sup>) and LLOQ (lowest limit of quantitation, 300/200 ng mL<sup>-1</sup>) for ZVD and NVP, respectively, were selected to perform different validation parameters.

### Sample pretreatment and extraction procedure

Drugs were extracted from plasma samples using a protein precipitation technique. Methanol was selected as the precipitating agent. Each plasma sample gave satisfactory values for recovery with a single extraction. The satisfactory result was obtained when 1:4 ratios of plasma and methanol were mixed thoroughly, vortexed at room temperature and centrifuged at 12000 rpm for 10 min at 4°C. The clear supernatant liquid was decanted, filtered through a 0.45  $\mu$ m syringe filter and injected (20  $\mu$ L) into HPLC system.

### **Limit of detection (LOD) and lowest limit of quantification (LLOQ)**

The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3. The lowest limit of quantification (LLOQ) was calculated to be the lowest analyte concentration that could be measured with a signal-to-noise ratio of 10. To determine LLOQ and LOD, plasma samples were spiked with decreasing concentrations of the analytes and analyzed. The LLOQ and LOD were observed 300 and 63 ng mL<sup>-1</sup> for ZVD while for NVP the values were 200 and 17 ng mL<sup>-1</sup>, respectively.

### **Linearity of method**

Calibration plots for the analytes in plasma were prepared by spiking drug-free plasma with standard stock solutions to yield concentrations of 300-9600 ng mL<sup>-1</sup> for ZVD and 200-6400 ng mL<sup>-1</sup> for NVP. The solutions were injected in replicates (n = 5) into the HPLC column while keeping the injection volume constant (20 µL). Calibration curves were constructed by using ratios of the observed analyte peak area versus concentration of analyte. Intercept, slope and correlation coefficient (r<sup>2</sup>) were determined by linear regression data analysis, which were then used to calculate the analyte concentration in each sample.

### **Precision and accuracy of method**

The inter-day, intra-day, analyst to analyst precision and accuracy of the assay were determined by assaying three QC samples and LLOQ in replicates (n = 5) for each drug. Precision was reported as percent standard deviation (% RSD) and accuracy was as % nominal concentration and % bias.

### **Recovery of zidovudine and nevirapine from plasma**

Recovery from plasma was determined for QC samples of each drug by comparing the peak area of each analyte after extraction with the

respective non-extracted standard solutions at the same concentration. The percentage of the drug recovered from the plasma samples was determined by comparing the peak height ratio after extraction with those of unextracted sample containing same concentrations of the drugs as in plasma.

### **Selectivity**

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. To evaluate the selectivity of the assay, blank samples of the appropriate biological matrix (plasma) were prepared from six different sources.

### **Stability of method**

Stability of drugs in biological fluids is a function of the sample storage conditions, chemical properties of the drug, matrix and the container system<sup>18</sup>. Blank samples were spiked with appropriate aliquots of diluted ZVD and NVP stock solutions to prepare LQC and HQC samples. The stability of drugs was evaluated under conditions likely to be encountered during actual sample handling and analysis. These samples were kept to evaluate different stability parameters such as stock solution stability (SSS, 6 h at room temperature), bench top stability (BTS, 12 h at room temperature), post processing stability (PPS, over the maximum time, i.e., from the completion of sample work-up to the completion of data collection), freeze and thaw stability (FTS, subjected to three freeze-thaw cycles of -20 °C during 24 h) and long-term storage stability (LTS, subjected to freeze storage at -20 °C during the entire period covered by the bioanalytical study, i.e., from the first day of sample preparation to the last day of sample analysis). For each concentration and storage conditions three replicates were analyzed. The concentration of ZVD and NVP after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

## RESULTS

At the chromatographic conditions selected for this method, the retention time for blank plasma (Fig. 2) and extracted peaks of ZVD and NVP (Fig. 3) were  $2.7 \pm 0.3$  min,  $5.2 \pm 0.3$  min and  $9.5 \pm 0.3$  min, respectively. The correlation coefficients for the linearity of the present method were 0.9997 and 0.9995 for ZVD and NVP respectively. The lowest limit of quantification and limit of detection were found to be 300 and  $63.0 \text{ ng mL}^{-1}$  and 200 and  $17.0 \text{ ng mL}^{-1}$  for ZVD and NVP, respectively. The recovery of the extraction procedure for ZVD was 95.39 %, 94.01 % and 89.51 % while for NVP, it was 94.93 %, 93.26 % and 92.13 % for HQC, MQC & LQC, respectively (Table 1).

The accuracy (% nominal conc.) for HQC, MQC, LQC and LLOQ, samples were found to be in the range 93.42 to 109.79 and 89.04 to 105.15 for ZVD and NVP, respectively. The accuracy data are shown in the Table 1 and were well within the acceptance limit. The precision data for HQC, MQC, LQC & LLOQ samples were analyzed in replicates ( $n = 5$ ) (Table 1). For intra-day, it was 1.47 to 8.59 % and 2.42 to 7.15 % for ZVD and NVP, respectively. For inter-day, the range was 1.93 to 9.38 % and 1.35 to 8.98 %, for ZVD and NVP, respectively, while for analyst to analyst precision, it was in the range 0.68 to 5.94 % and 1.79 to 6.82 % for ZVD and NVP, respectively.

**Table 1:** Accuracy, precision and mean recovery data for the developed method

QC Samples	Accuracy		Precision			Mean Recovery (%)	
	% N.C. <sup>a</sup>	% Bias	Intra day	Inter day	A-to-A <sup>b</sup>		
ZVD <sub>c</sub>	LLOQ <sup>d</sup>	104.38	4.38	8.59	9.38	4.50	-
	LQC <sup>e</sup>	97.70	-2.30	2.34	7.03	5.94	91.39
	MQC <sup>f</sup>	109.79	9.79	5.51	4.37	2.62	95.01
	HQC <sup>g</sup>	93.42	-6.58	1.47	1.93	0.68	89.51
NVP <sup>h</sup>	LLOQ	89.08	-10.92	6.26	5.92	5.50	-
	LQC	98.44	-1.56	7.15	8.98	3.72	90.93
	MQC	106.15	6.15	2.42	2.67	6.82	93.26
	HQC	100.80	0.80	4.13	1.35	1.79	92.13

<sup>a</sup> % Nominal concentration; <sup>b</sup> analyst to analyst; <sup>c</sup> zidovudine; <sup>d</sup> lowest limit of quantitation; <sup>e</sup> low quality control; <sup>f</sup> medium quality control; <sup>g</sup> high quality control; <sup>h</sup> nevirapine.

**Table 2:** Stability of the developed method at various conditions

Stability conditions	Zidovudine				Nevirapine			
	LQC		HQC		LQC		HQC	
	% N.C. <sup>a</sup>	% C. <sup>b</sup>	% N.C.	% C.	% N.C.	% C.	% N.C.	% C.
SSS <sup>c</sup>	92.68	4.94	98.45	-1.13	103.73	2.83	92.69	-5.31
BTS <sup>d</sup>	95.51	-2.23	88.60	-7.52	97.15	6.25	94.92	-1.63
PPS <sup>e</sup>	103.53	5.21	99.35	-3.23	91.59	-9.69	99.40	1.11
FTS <sup>f</sup>	96.58	3.51	91.96	-5.43	99.57	8.95	104.10	5.49
LTS <sup>g</sup>	107.25	8.28	102.74	6.26	109.50	11.97	105.60	-0.51

<sup>a</sup> % Nominal concentration; <sup>b</sup> % change; <sup>c</sup> stock solution stability; <sup>d</sup> bench top stability; <sup>e</sup> post processing stability; <sup>f</sup> freeze & thaw stability; <sup>g</sup> long term stability

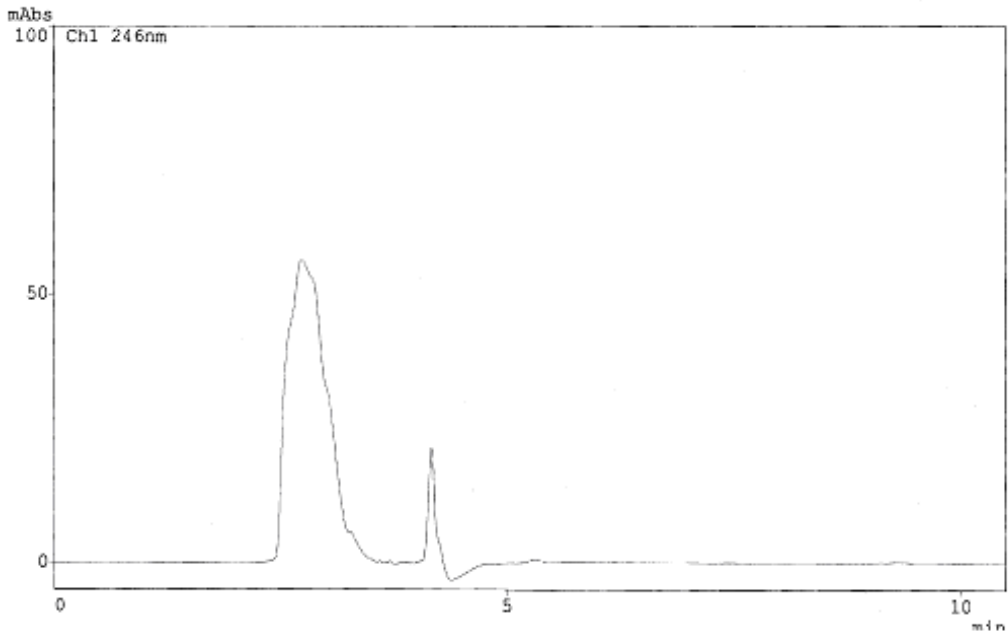


Fig. 2: Representative chromatogram of blank plasma

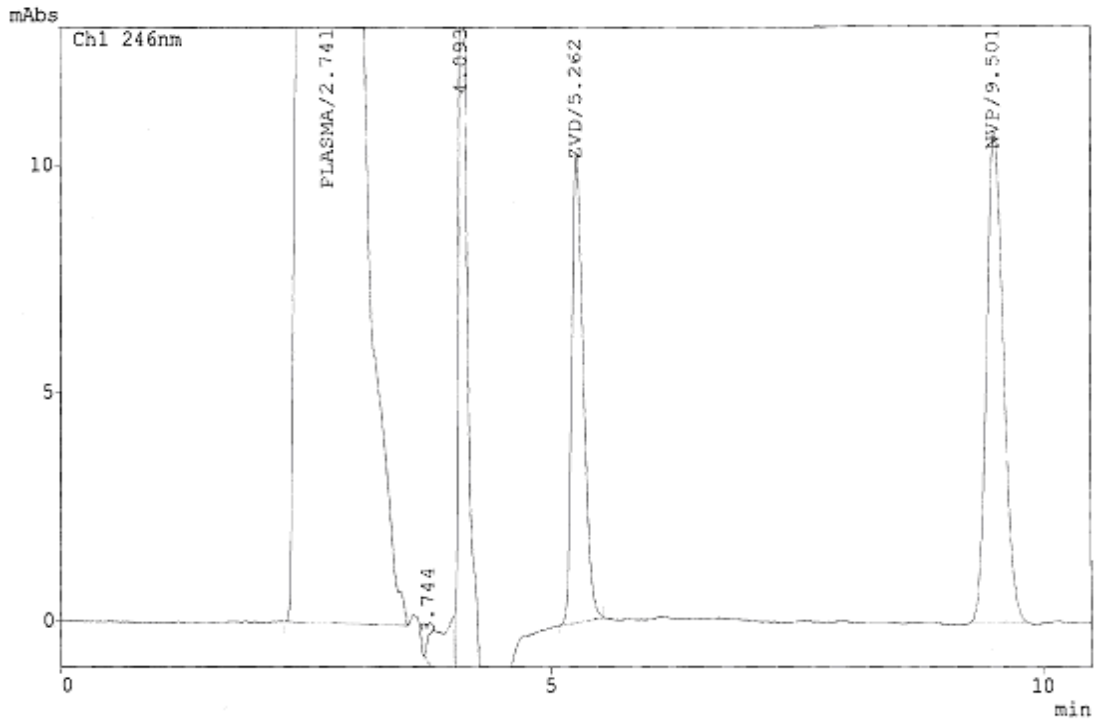


Fig. 3: Extracted chromatogram of zidovudine (ZVD) and nevirapine (NVP)

The stability test results (% change) for ZVD at 6000 and 600 ng mL<sup>-1</sup> were [(4.94 and -1.13 for SSS), (-2.23 and -7.52 for BTS), (5.21 and -3.23 for PPS), (3.51 and -5.43 for FTS), (8.28 and 6.26 for LTS)] and for NVP at 4000 and 400 ng mL<sup>-1</sup>, they were [(2.83 and -5.31 for SSS), (6.25 and -1.63 for BTS), (-9.69 and 1.11 for PPS), (8.95 and 5.49 for FTS), (11.97 and -0.51 for LTS)] as illustrated in Table 2. The result of % change demonstrates that stability testing of ZVD & NVP were within the acceptance range of  $\pm 15\%$  deviation from the nominal concentration.

## DISCUSSION

The present study describes a sensitive, accurate, and reproducible HPLC method for the determination of ZVD and NVP in human plasma. Here, we used a simple protein precipitation with a small quantity of plasma and precipitating agent, which considerably reduced the sample processing time. There is no need to use an internal standard as recovery of the method was found to be more than 90 %. The mobile phase used in this study was economical, simple and the assay was entirely isocratic involving only two stable solvent components, water and acetonitrile; thus the chances of column damage were negligible, which gives the column a longer life span.

In order to obtain the most suitable wavelength for the detection of ZVD and NVP, a UV spectrum was obtained. The UV spectrum revealed that maximum absorbance peaks were 266 nm for ZVD and 283 nm for NVP but the selected wavelength was 246 nm. Although this was not the maximum absorption wavelength for either of the drugs, it, nevertheless, provided good signal to noise ratio and selectivity.

The retention time for ZVD and NVP were  $5.2 \pm 0.30$  min &  $9.5 \pm 0.30$  min, respectively. Blank plasma samples did not give any peak at the retention time of these drugs. The LLOQ value, 300 and 200 ng mL<sup>-1</sup> for ZVD and NVP, respectively, allowed us to avoid the

use of expensive and sensitive detectors. The calibration curve derived from eight different concentration points ranging from 300 to 9600 ng mL<sup>-1</sup> for ZVD and 200 to 6400 ng mL<sup>-1</sup> for NVP, showed a linear relationship between area under curve (AUC) and concentration. The mean extraction recovery of 92.97 % for ZVD and 93.44 % for NVP is satisfactory since no comprehensive method of extraction was used. The accuracy and precision data are well under the acceptance limit ( $\pm 20\%$  for LLOQ, and  $\pm 15\%$  for LQC, MQC and HQC). The stability data of analytes show that the drugs are stable in plasma under the varying conditions of the tests.

## CONCLUSION

A simple, sensitive, isocratic, reverse phase HPLC-UV assay was developed and validated for the simultaneous analysis of zidovudine and nevirapine in human plasma. The small volume plasma sample preparation involved rapid protein precipitation extraction, making it widely applicable for HIV clinical therapeutic drug monitoring programs. The results of this study indicates that the method would be applicable to toxicokinetic, pharmacokinetic, bioavailability, and bioequivalence studies. This HPLC method should also be useful for analyzing plasma concentrations of the drugs evaluated as well as pharmacokinetic parameters in HIV-infected patients for the purpose of evaluating clinical pharmacology and clinical therapeutic drug monitoring (TDM) programs for these drugs in HIV studies.

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