Available on line www.jocpr.com

Journal of Chemical and Pharmaceutical Research

J. Chem. Pharm. Res., 2010, 2(2): 431-439

ISSN No: 0975-7384

Bioanalytical method for determination of Nevirapine *in-vivo* in resource constrained laboratories

Omary M. S. Minzi[*1] and Eliford Ngaimisi[2]

[1,2]*Unit of Pharmacology & Therapeutics, School of Pharmacy, Muhimbili University of Health and allied Sciences P.O BOX 65013, Dar es Salaam Tanzania*

Abstract

Simple high-Performance Liquid Chromatographic method for determination of Nevirapine in human plasma is hereby reported. Carbamazepine was used as internal standard (IS). The internal standard and the drug were extracted into basified di-isopropyl ether, dried with a current of air using hair drier. The dried sample was reconstituted with 120 µl of mobile phase followed by injection of 90 µl of the reconstituted sample into the chromatograph. A reversed phase column C18 was used and the mobile phase consisted of 250ml acetonitrile and 800 ml phosphate Buffer (pH 7.5). Detection was achieved at 282nm and both NVP and IS were well separated from endogenous substances. The method involves single step extraction procedure and applies a commonly available internal standard. It is suitable for use in laboratories of developing countries as it applies readily available carbamazepine as internal standard, C-18 column and does not require nitrogen evaporation system and high speed centrifuges whose availability may be an issue in resource constrained laboratories.

Key words: High-Performance Liquid Chromatographic method, Nevirapine, Human plasma, Carbamazepine.

Introduction

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used as one of antiretroviral drugs (ARV) in treatment guidelines of many countries where HIV-1 is

predominant. The drug is combined with other 2 nucleoside analogues resulting into an enhanced inhibition of reverse transcriptase enzyme (RT) which is a key enzyme responsible for viral replication. NVP noncompetitively binds to the allosteric site of the RT enzyme thus impairing the reverse transcription process[1,2].

For successful treatment outcomes, therapeutic plasma NVP levels are required, with low levels being associated with treatment failure and emergence of NVP resistant strains. Plasma NVP levels depend on drug bioavailability and drug clearance both of which vary among individuals. Genetic polymorphism involving metabolizing enzymes has been implicated to cause inter-individual variability in the bioavailability of some drugs[3,4].

Due to plasma levels variation over a time within a patient and also due to high risks of developing drug resistance, there is a need of continuous monitoring the drug kinetics in patients undergoing NVP based HAART for the purpose of ensuring that the drug levels are maintained within therapeutic plasma concentration ranges. Resistance to NNRTIs develops very quickly especially when sub therapeutic drug levels are maintained at the site of action for a long time[5].

To be able to monitor plasma levels of NVP in patients, a suitable analytical method is needed. The biggest challenge of pharmacokinetic studies in patients undergoing HAART, is the high number of additional drugs other than ARVs which a patient uses. For a successful determination of NVP in patients cotreated with a high number of drugs, a very selective analytical method is needed.

Recently, high pressure liquid chromatographic methods involving plasma sample precipitation were reported. HPLC methods involving precipitation procedures are simple, cheap and are less time consuming. Such methods could be very useful in analysing a huge number of samples particularly in cases where sample injection is done manually. Unfortunately, precipitation does not completely free the plasma off proteins and other endogenous substances. To circumvent this, high speed centrifugation of up to 10,500 g is needed. We tested some of the published protein precipitation methods in our laboratory but none of them were completely satisfactory for our purpose as NVP was always poorly resolved from endogenous substances[6-8]. Recently a method for determination of nevirapine and efavirenz by precipitation method applying a mobile phase at pH (11.7) has been reported[6]. Highly alkalinized mobile phases are not user friendly for the life span of reversed phase columns. Methods applying liquid-liquid extraction have superiority over precipitation methods in terms of selectivity and column efficiency.

A simple extraction method using 3-isobutyl-1-methyl xanthine as internal standard (IS) was recently reported[9]. We wanted a simple and selective method which could enable determination of plasma levels of NVP using readily available internal standard and C18 columns.
The method we hereby report applies single step extraction procedure and carbamazepine (CBZ) as IS.

Materials and Methods

Experimental

2.1. Chemicals

NVP, Stavudine, Lamivudine and Zidovudine reference standards were obtained from The Tanzania Food and Drug Agency (TFDA) analytical laboratory in Dar Es Salaam, as generous donation by Danstan Shewiyo. CBZ as internal standard was a gift from Margarita Mahindi an analytical engineer at the laboratory of Department of Medical Sciences and Technology, Karolinska Institute (KI), SE-141 86 Huddinge, Stockholm, Sweden. TMP and sulfamethoxazole (SM) were previously obtained from Sigma Chemical Co. (St Louis, MO, USA). Solvents and chemicals (HPLC or analytical grade) were purchased locally but were initially ordered by a local company from BDH Company (Darmstadt, Germany).

2.2.Chromatographic system

The chromatographic system used was devised with an auto sampler model SIL-20A (Shimadzu Corporation Kyoto Japan). A Shimadzu solvent delivery module LC-20AT VP pump (Shimadzu Corporation Kyoto Japan) and a Shimadzu UV-Visible spectrophotometric detector SPD-10A (Shimadzu Corporation Kyoto Japan) was used.

A Microsorb®, C_{18} column (150x4.6 mm I.D, 5 µm particle size (Varian, Middelburg, The Netherlands) was used and the mobile phase consisted of 250ml acetonitrile and 800 ml phosphate Buffer pH 7.5).

Elution was carried out at room temperature using a flow rate of 0.8 ml/min and detection was achieved at 282 nm. Method development was carried out in the Unit of Pharmacology and Therapeutics, School of Pharmacy, Muhimbili University of Health and Allied Sciences (MUHAS).

2.3. Preparation of standard solution

Stock solutions were made by dissolving NVP and IS in about 5 ml methanol in 10 ml volumetric flasks followed by sonication for 10 minutes. Dilution to volume was made using deionised water. For calibration curves, NVP 8.6µg/ml working solution and IS 11.6µg/ml working solutions were used. The quality control samples which were also used for method validation were prepared in similar way and had concentrations of 1.2, 2.4 and 4.8 µg/ml. The stock solutions, working standards and quality control samples were always kept in a deep-freezer at -20^{o}C.

2.4.Sample preparation and analytical procedure

Drug free plasma (blank) was spiked with NVP and IS to obtain final concentrations of 1.8 -7.2 µg/ml and the samples were used in developing and evaluating the method. Aliquots of 100 µl plasma were frozen into 4ml polypropylene tubes at -20˚C until assay.

To 4ml propylene tube containing 100µl spiked plasma 25 µl IS with concentration of 11.6 µg/ml (for NVP concentration ranging between 0.5-4.0 µg/ml and 34.8 µg/ml IS for NVP concentrations ranging between 5.4-7.2 µg/ml) was added followed by 200 µl carbonate buffer pH 9.8. The mixture was vortex mixed for 20s and the drugs were extracted from plasma

samples using 500µl of di-isopropyl ether by gentle shaking on a reciprocal shaker for 10 minutes. After centrifugation for 10 min at 3500 rpm, 350µl of the upper organic phase was transferred to a new tube and was dried with a current of air. The dried sample was reconstituted with 120 µl of the mobile phase. Into the chromatograph, 90µl of resultant solution were injected.

2.5.Calibration curves

The standard curves were prepared by duplicate analysis of 100µl plasma spiked with 0-40µl working solution of NVP to achieve a concentration range of 0-3.44 µg/ml.

Two quality control samples in duplicate (low and high level) containing NVP at the concentrations of 2.4 and 4.8µg/ml were always included in each analysis. For higher NVP plasma concentrations a calibration curve range of 1.72-6.88µg/ml was prepared. The concentrations of IS used are as described above. The resulting peak area ratios between the internal standard and the analyte were plotted versus concentrations.

2.6. Extraction recovery

To check the extraction recovery, the lower and higher level quality control samples were used. NVP and IS from 100 µl spiked plasma samples were extracted and assayed as described previously. Peak areas from extracted drugs and directly injected solutions were compared in order to determine the extraction recovery.

2.7. Precision and accuracy

Known amounts of NVP were added to aliquots of 100 µl blank plasma to achieve a known concentration. The samples were extracted and analyzed as described earlier both in one series (within assay precision and accuracy) and on different occasions (between assay precision and accuracy). The standard deviations and coefficients of variation were calculated. The results are presented in table 1 and 2.

Table 1: Intra- and inter-day precision for the determination of NVP in human plasma (n=6)

Intra-day validation (n=6)				Inter-day validation(n=8)		
Nevirapine	Spiked plasma	Mean determined	CV %	Spiked plasma	Mean determined	CV %
Level	Conc. µg/ml	conc. µg/ml		Conc. µg/ml	Conc. µg/ml	
Low	0.80	0.84	9	0.80	0.72	9.6
Medium	2.40	2.25	5.1	2.40	2.20	5.0
High	4.80	4.93	4.2	4.80	4.50	3.5

Table 2 : Inaccuracy for the determination of nevirapine in human plasma (plasma samples n=6)

Theoretical plasma	Observed plasma	Inaccuracy
conc. (µg/ml)	conc. (µg/ml)	% (n=6)
1.80	1.65	9.9
2.40	2.22	3.4
7.20	7.11	2.9

2.8.Specificity

Standard solutions containing sulfamethoxasole, trimethoprim, fluconazole, Zidovudine, Lamivudine, Stavudine were injected into the chromatograph. The drugs were spiked in blank plasma to achieve their therapeutic concentrations and extracted as described previously. The extracted samples were injected into the chromatograph as described previously and peaks were monitored for interference. Endogenous substances were also monitored for interference.

2.9. Stability

The stability test of NVP plasma samples was not performed as it has been reported elsewhere[6,10].

2.10. Samples from a patient

Samples from a single patient undergoing antiretroviral treatment using NVP based HAART were also used. The patient has been taking an oral dose of 1 tablet which contains 150 mg Lamivudine, 30 mg Stavudine and 200 mg nevirapine as fixed dose combination formulation twice a day for the past 6 months Venous blood samples 1.0 ml were obtained from this patient and the collected blood sample was immediately centrifuged at 3500 rpm for 15 minutes to get plasma. The obtained plasma sample was stored as previously described until assay. The sample were analyzed on 5 separate days as described above.

Results and Discussion

The chromatographic system used exhibited a good separation for NVP and CBZ (the IS) after injection of extracted spiked samples and samples from patients. The tested drugs did not show any interference and the endogenous substances were well separated.

The separation chromatogram of standard solution containing a mixture of NVP and CBZ as IS is shown in Figure 0-1.

The retention times for NVP and IS were always 4.5 and 9 minutes respectively. The endogenous substances were well separated from the peaks of NVP and IS as shown in fig 0-2.

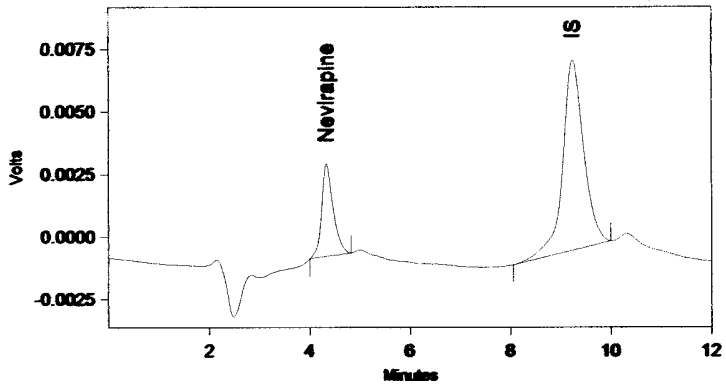

Figure 0-1 Separation chromatogram of NVP and IS in an aqueous mixture of the 2 substances

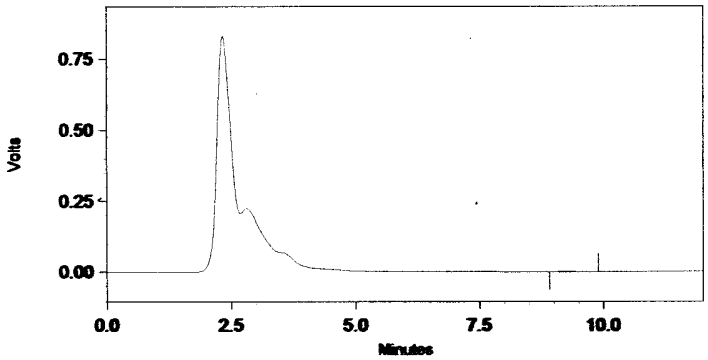

Figure 0-2 Extracted blank plasma from human blood

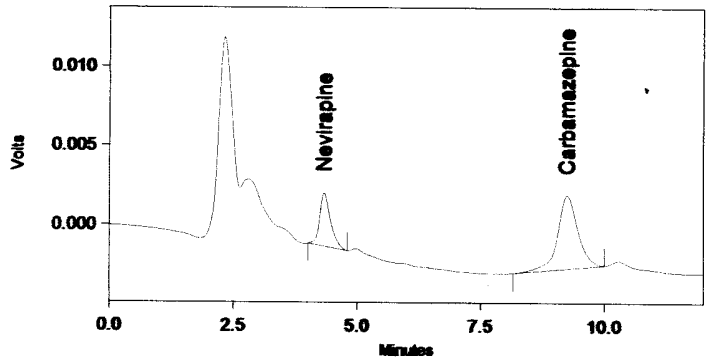

Figure 0-3 A separation chromatogram of NVP and IS extracted from spiked human plasma

Figure 0-3 and 0-4 show the chromatograms obtained from plasma samples spiked with NVP and IS and from a patient who has been on ART for 6 months respectively.

By careful inspection of the chromatograms, it can be seen that the retention times of NVP and IS in spiked samples and those obtained from the patient corresponded well to those obtained from a standard solution containing the 2 substances.

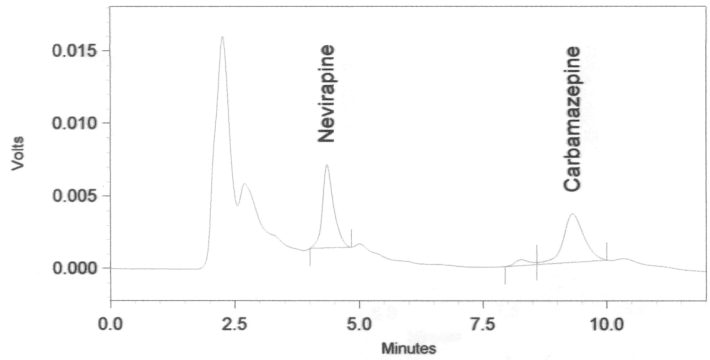

Figure 0-4 A separation chromatogram of NVP and IS extracted from plasma sample of an HIV infected patient undergoing NVP based HAART for more than 6 months

The calibration curves obtained from NVP spiked plasma samples in the concentration ranges studied were always linear. The correlation coefficients of calibration curves obtained were always in the range of 0.992-0.997 in all runs.

Intra and inter-day variations were always ≤10% demonstrating that the precision of the method is good under the selected conditions. Intra- and inter-day precision for the determination of NVP in human plasma (n=6) in plasma samples at concentration range of 0.8-4.8µg/ml always gave coefficients of variation ≤10% ranging between 3.5-9.6 indicating good precision of the method (table 1). The limit of quantification was 0.6µg/ml) with intra- and inter-assay variations of ≤10%. The Extraction recoveries for NVP and CBZ were 70% and 84% respectively.

Accuracy of the method was tested using spiked samples with NVP at concentrations of 1.8, 2.4 and 7.2µg/ml. The concentrations of NVP observed in relation to the theoretical values were always in the range of 90-110% indicating good accuracy of the method (table 2). The calculated concentrations of the quality control samples used in the analysis always gave results ranging between 90-110% of the expected concentration confirming the reliability of the method.

There was no interference observed by the tested drugs and endogenous substances did not show any interference on the peak of NVP and IS.

The mean nevirapine plasma levels determined from 5 runs of samples obtained from patient taking antiretroviral treatments for more than 6 months were 5.59±0.5 µg/ml respectively.

Methods which involve multi-step step extraction procedure are time consuming and in each step a significant loss of analytes is experienced ending up with poor recovery and low sensitivity. The method hereby reported involves a simple single step extraction process followed by sample drying with a current of air using a hair drier and injecting the reconstituted sample with mobile phase. The method showed good recovery, precision and selectivity with limit of quantification of 0.6µg/ml at a coefficient of variation of ≤10%. The coefficient of correlation of the method always ranged between 0.992-0.997 indicating good linearity of the method.

HIV infected patients undergoing treatment with highly active antiretroviral treatment also use co-trimoxasole for prophylaxis of pneumocystic carinii pneumonia. Fungal infections in HIV infected patients are also very common and the drug of choice is fluconazole. These drugs were tested for interference and none of them interfered with NVP peak or the IS. Only trimethoprim and sufamethoxasole peaks were found very close to the peak of NVP but were not detected after extraction confirming the selectivity of the method. However, it is important to completely avoid transferring an aqueous layer in the last step before drying as this will pick up huge number of endogenous substances which co-elute with NVP peak. It is the most important step in this method and we have clearly indicated that only 350µl of the upper organic phase and no more must be pipette and transfered to a new tube and dried with a current of air. We used a hair drier and the flow of air was adjusted at room temperature to avoid decomposition of substances.

Methods developed using spiked samples need to be tested using samples obtained from human volunteers since *in vitro* results do not always reflect the *in vivo* behavior of some drugs. Some drugs like amodiaquine are highly metabolized and may be not detected in blood shortly after drug administration even though in spiked samples they gave good recovery[11]. To ensure validity of our method, venous blood was collected in the morning pre-dose from a patient who has been using NVP based HAART and a mean value of 5.59±0.5 µg/ml NVP plasma concentration was obtained indicating the validity of the method in producing reliable analytical results from samples obtained in the field. The steady state trough plasma concentration of NVP in patients has been reported to range between 4.0-6.5 µg/ml[12].

We have developed a rapid and selective analytical method for determination of NVP from plasma samples. The method applies small volume of blood samples (0.5-1ml) as the plasma volume required for NVP determination is only 100µl. The developed method is a simple, one step extraction procedure giving good peak resolutions and applies a readily available IS. The method is highly selective enough to enable determination of NVP in samples obtained from patients undergoing ART and other treatments of common AIDS related illnesses.

Acknowledgement
This study was financially supported the Swedish Agency for Research Cooperation with Developing Countries, (Sida) which is highly acknowledged. We also thank the patient who volunteered to give his blood for testing the performance of our method.

Reference

[1] Zanoli S, Gemma S, Butini S, et al. *Biochem Pharmacol*. **2008**, 76:156-68.
[2] Fattorusso C, Gemma S, Butini S, et al. *J Med Chem.* **2005**, 48:7153-65.
[3] Boulle G, Van Cutsem K, Cohen, K et al. *JAMA.* **2008**; 300:530-539.
[4] Rotger M, Colombo S, Furrer H et al. *Pharmacogenet Genomics* **2005**; 15: 1-5.s
[5] Delaugerre C, Rohban R, Simon A. *J.Med. Virol.***2001**; 65: 445 – 448**.**
[6] Kappelhoffa BS, Rosinga H, Huitemaa A, Beijnena JH. *J Chromatogr B,;* **2003**; 792: 353–362.
[7] Lopez RM, Pou L, Gomez MR, Ruiz I, Monterde J. *J Chromatogr B,* 751: 371-376.
[8] Van Heeswijk R, Hoetelman R, Meenhorst P, Mulder-JW, Beijnen J. *J Chromatogr B,* **1998**; 713: 395-399.

[9] Ramachandran G, Hemanthkumar A, Kumaraswami V, Swaminathan S. *J Chromatogr B,* **2006**; 843: 339–344.
[10] Bennetto C, King JR, Turner ML, Stringer JS, Acostal EP. *Clin Chem*, **2004**, 50: 209-211.
[11] Hombhanje FW, Hwaihwanje I, Tsukahara T et al. *Br J Clin Pharmacol.* **2005**, 59:298-301.
[12] Nellen JF, Damming M, Godfried MH et al. *HIV Medicine*, **2008**, 9:234-238.