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ORIGINAL ARTICLE

Use of Hair Samples for Monitoring of Antiretroviral Therapy Adherence

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ABSTRACT

Introduction: Measurement of antiretrovirals (ARVs) drug concentration in biological matrices such as blood and urine has been used previously for monitoring adherence. Unfortunately, they only reflect ARV doses taken within 1 to 2 days of sampling. Hair testing has become the most preferred tool to determine chronic exposure to some drugs, especially drugs of abuse, because of its long detection window.

Objective: This study, evaluated the utility of hair samples in therapeutic drug monitoring (TDM) as an indicator of ART adherence.

Methods: This study used nevirapine (NVP), an ARV integral component of the first line ART in Kenya, for many years. Matched hair and blood samples were obtained from 234 and 328 consenting HIV patients on first line ART with virologic failure (viral load >1000 copies/mL) and suppressed viral load (VL<1000 copies/mL) respectively. The ARV plasma and hair concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: The calculated median; interquartile range (IQR) of NVP levels in hair and plasma samples were 36.8ng/mL and 19.32ng/mL respectively. There was no significant difference between the level of NVP in hair and matched plasma samples (Wilcoxon signed rank test; Z = -0.93, $P > 0.05$).

Conclusion: The study has demonstrated that analysis of ARV drugs in the hair can determine drug exposure as an alternative to conventional plasma drug analysis, especially in our settings where laboratory facilities and skilled personnel to do phlebotomy are few or lacking.

INTRODUCTION

The treatment of HIV is complex, and for a successful clinical outcome, adherence to the Antiretroviral Therapy (ART) must be considered. ART is a critical component in reducing the HIV epidemic.[1] Globally, progress has been made in the HIV research and treatment cascade. Antiretroviral therapy has been used globally by 67% (25.4 million) of people living with HIV at the end of 2019.[2] In Kenya, 74% (1,112,254) of adults and 73% (71,500) of children in need of ART were receiving antiretroviral (ARVs) by the end of 2019.[3]

A remarkable proportion of these patients (68%) were virally suppressed.[2] At the time of the study, the first-line ART in Kenya for children, youth, and adults included two nucleoside reverse transcriptase inhibitors (NRTIs) plus one non-nucleoside reverse transcriptase inhibitor (NNRTI). The common NRTIs were zidovudine (AZT), stavudine (d4T), tenofovir (TDF), or lamivudine (3TC), NNRTIs were nevirapine (NVP) and efavirenz (EFV).[4] The first line of ART was a combination of one NNRTI (NVP or EFV) and two NRTIs (3TC, AZT).[4]

Analysis of ARVs in the blood of PLWH is a direct technique used to monitor PLWH's adherence to ART. Measurement of ARV levels in PLWH on ARVs is the foremost step in monitoring ARV drugs' adherence.[5] Unfortunately, the whole process involving the analysis of drugs in blood is expensive and unaffordable in most resource limited settings.

Hair is becoming an essential biological specimen for drug testing besides urine and blood. Cocaine, opiates, and amphetamines have been analysed in the hair.[5] The hair sample is obtained quickly, does not require specialized personnel to collect, and is stored at room temperature while awaiting analysis in the laboratory.[5] These features provide obvious cost and feasibility advantages for hair collection and storage over plasma samples. Further, hair analysis provides an advantage over blood or urine testing in assessing long term adherence.[5,6] This study, therefore, evaluated the utility of hair samples in TDM as an indicator of ART adherence to be used as an alternative method to ARVs analysis in blood. Hair analysis represents a cheaper option and is easier to adapt to the hospital setting for monitoring adherence to ART among people living with HIV

(PLWHIV) in low resource settings or where skilled personnel to perform plasma analysis are few or lacking.[7]

METHODS

Study Design and Site

A cross sectional study design was used in the sample collection. The consenting participants were recruited at two regular HIV care clinics Nairobi and Kisumu Counties. This study recruited patients who had health records available and had been on ART treatment for at least 12 months and were deemed to have attained steady state drug plasma concentrations. The PLWHIV with viral loads of more than 1000copies/mL was 81.7% of the study participants, while those whose viral load was less than 1000copies/mL after 12 months of being on a first line ART regimen were 62.2% of the study participants. A total of 268 and 145 participants were recruited from the Nairobi and Kisumu counties, making a total of 413 participants. The levels of HIV viral loads were extracted from the participants' medical folders.

The Inclusion Criteria for Cases and Controls

Eligible study participants were PLWHIV aged above 18 years on nevirapine (NVP) for over 12 months who attended the two HIV treatment care clinics in Kisumu and Nairobi and were willing to give written informed consent voluntarily. In addition, eligible study participants were required to have viral load results at month 12 of treatment. Participants were stratified into two groups, those with non-viral suppression with HIV-1 RNA viral load (VL) >1000 copies per ml of blood after being on treatment for a period of not less than 12 months and those with suppressed viral loads with HIV-1 RNA viral load (VL) <1000 copies. Participants' hair samples were taken from those who had not used hair dyes or permanent hair products in the previous three months and had hair length of not less than 1cm long.

Sample Collection

Plasma sample

The Blood was drawn from the vein located on the inside of the elbow of the participants. Approximately 5ml of whole blood was collected in plasma preparation tubes (PPT) with ethylenediaminetetraacetic acid (EDTA). The blood was centrifuged to obtain plasma aliquots and stored at -80˚C. The plasma samples were transported on dry ice to the KEMRI Centre of microbiology laboratory for storage and laboratory testing then stored at -80˚C at the central laboratory until NVP extraction.[8]

Hair Samples

The hair samples of the participants were cut with scissors from the human occipital scalp. The hair samples were covered in aluminums foil and placed in zip lock plastic bags with labels. They were delivered to the KEMRI HIV laboratory in sealed envelopes at room temperature and batched for testing.[9]

Materials and Reagents

Jomo Kenyatta University of Agriculture and Technology generously donated the NVP drug and carbamazepine as an internal standard (IS). The used HPLC grade acetonitrile, methanol (MeOH), analytical grade trifluoroacetic acid (TFA), Di-Methyl ether, ethyl acetate and ammonium acetate were purchased from Sigma Aldrich/Merck.

Preparation of Standard Solution for hair samples

A stock solution of 1mg/ml of both nevirapine and internal standard carbamazepine were prepared in methanol. The internal standard solution was prepared by diluting the stock solution with methanol to give 100ng/mL solution. Standard solutions were prepared by subsequent dilution of the stock solution with methanol to acetonitrile (20:80, v/v) concentrations ranging between 10ng/mL to 500ng/mL, with 100µL of carbamazepine was added to each solution.

Preparation of Standard Solution for Plasma Samples

A stock solution of nevirapine was prepared at a concentration of 1mg/ml in methanol. The working standard solutions were prepared in water: Acetronitrile (20:80, v/v) from stock solution. Standard solutions were prepared by subsequent dilution of the stock solution with methanol to concentrations ranging between 10ng/mL to 1000ng/mL.[10]

Extraction of NVP from Hair Samples

Hair samples of 10mg from each participant were weighed, cut into approximately 3mm, and placed into a glass test tube (16mm diameter x 125mm height). Internal standard (IS) was added to each sample at a 100ng/mL concentration. The extraction of NVP from hair was done by adding 2mL of methanol/trifluoroacetic acid (9/1, v/v ratio) solution to the samples and shaking in a reciprocal shaker for 16 hours.[11] The organic solvent was then evaporated to dryness by nitrogen gas. The extracted drugs were cleaned up further by liquid-liquid (Liq-Liq) extraction as described below. Briefly, 0.5ml of 0.2M sodium phosphate buffer (pH 9.4) was added to the sample and vortex mixed for 30 seconds. Three (3mL) dimethyl ether/ethyl acetate (1:1) measuring was added, and the mixture vortexed three times, each for 1 minute. This was then centrifuged at 3000 rotations per minute (rpm) for 10 minutes. The sample was frozen in dry ice before transferring the supernatant layer to a fresh test tube (13mm diameter x 100mm height). One hundred microliters (100µL) of 1% trifluoroacetic acid in methanol were added to the supernatant layer, and the sample evaporated to dryness using nitrogen gas. Reconstitution was done with 0.5ml of acetonitrile/water 1:1, and vortex mixing followed, each for 30 sec. The extracts were filtered using 0.45µm microfilters and then transferred into auto-sampler vials, and a 10µL aliquot was injected into the LC-MS/MS for analysis.

Extraction of from Plasma Samples

The protein precipitation method was used to extract NVP from plasma samples, followed by a liquid-liquid extraction process.[7] Plasma samples (0.2mL) were transferred to 2mL of Eppendorf tubes, and 0.2mL of organic solvent (50mM ammonium acetate solution: acetonitrile in the ratio1:6) was added for protein precipitation then vortex for 3 minutes. One (1mL) ethyl acetate was added to the above samples to extract analytes into the organic layer and hold the endogenous plasma materials water-soluble in the aqueous layer to decrease the matrix influence. The resulting samples were vortexed for 5 minutes and centrifuged for 10 minutes at 4°C at 12,000 rpm. The

supernatant was transferred into a new glass tube and evaporated at 37°C under nitrogen. After evaporation, a second ethyl acetate extraction was performed. For the dried extract, the organic layer was added and evaporated. Aliquots of 500µL made up of HPLC grade water and acetonitrile in a ratio of 1:15 of acetonitrile was used to reconstitute the residue, and 10µL aliquot was injected into the LC-MS/MS systems.[8]

NVP Quantification using LC-MS/MS

A very sensitive, selective, and reproducible LC-MS/MS method developed by[11] was validated and used to analyse hair samples and a LC-MS/MS method developed by [8]was validated and used to analyse plasma samples. The LC/MS/MS analysis was conducted under positive MRM mode, and the compounds were identified using ion mass and retention times. The triple quadrupole 6410 LC/MS and a Agilent HPLC system (Agilent) 1100 A were used in the study. The columns used for separation were Kinetex Evo C-18 (3mm x 100mm), 5µm for hair samples, and C18 4.6mm by 150mm), 5µm particle size for plasma samples. Mobile phases' flow rate was 0.45mL/minute. The temperature of the autosampler was 15°C, and the injection volume was 10µl. The mobile phase was made of 0.1% formic deionised water (mobile phase A) and 0.1% formic Acetonitrile (mobile phase B). The isocratic mode was used in the ratio of 20:80 of A to B; the column's temperature was set at 30°C. The total run time for LC was 10 minutes. The analytes and carbamazepine (CBZ) detection were done on a triple quadrupole mass spectrometer, equipped with an electro ion spray ionisation mode and positive ion mode. Masslynx software was used to control all LC and MS parameters. Transition ions and optimal conditions used to obtain a relative abundance of product ions were as follows: The cone voltage during the analysis of NVP and CBZ were 36V and 32V respectively. The collision temperatures were 25 and 20 °C for the analysis of NVP and CBZ respectively.

Multiple reaction monitoring (MRM) was used to quantify both NVP and IS. Precursor ions and product ions were optimized by directly injecting 1000ng/ml solutions of NVP and IS into the MS in a suitable mass range, respectively, in positive and negative polarity modes using the atmospheric technique. The highest intensity for [M+H] + ions was found in positive mode for NVP and IS, both accepted protons. The compounds and their relative molecular mass, precursor ions, and product ions are 267>226 and 237>197 for NVP and CBZ respectively. The LC-MS/MS method has high selectivity for the only precursor. The product ions of the analyte of interest monitored by MRM mode supported the precursor m/z and its fragment m/z (MRM transition) for every analyte.

Statistical Analysis

The concentrations of ARVs in hair samples were back calculated from the calibration curve of the NVP/CBZ ratio against concentration. The concentrations of ARVs in plasma samples were back calculated from the calibration curve of the NVP area against concentration. The Kolmogorov-Smirnov test and the Shapiro-Wilk test were used to determine the normality of data. The Wilcoxon W Test was used to evaluate the hypothesis. The test involves ranking the absolute paired differences between of plasma and hair.

Ethical approval

This study was approved by the KEMRI Scientific and Ethics Review Committee SERU Protocol Number 3214. Prior consent was sought from the specific hospitals where the sample collection took place in the two County Governments of Kisumu and Nairobi. Participants were assured that all information obtained from them would be treated with maximum confidentiality, and no names would be published in any report. All biological waste was collected in biohazard bags, followed by incineration.

RESULTS AND DISCUSSION

Demographic Characteristics of the Study Population

Study Population (Table 1) shows the demographic characteristics of study participants. A total of 413 participants on NVP as part of their ARV regimen were recruited for the study. The women participants contributes 58.4% of the study sample size. The median age was 41 years and the median duration of treatment was 6 (range, 3-11) years. Hair samples of 223 participants were collected and presented to the laboratory. Only 105 participants donated enough hair quantity >10mg since some were willing to donate their hair samples, but their hair was very short. The 328 plasma samples were collected and submitted to the laboratory to quantify nevirapine (NVP). However, only 308 plasma samples were analysed for NVP level because some participants who donated blood samples were reluctant to grant their hair samples. Some donated hair and were unwilling to donate blood samples. Therefore, participants with the matched plasma and hair samples were only 94 which represented 22% of the participants. 51.4% of participants who doneted their samples for analysis missed taking current ARVs the whole day or more (Table 1).

Determination of Nevirapine Levels in the Plasma and Hair Samples

The *P* value of the Shapiro-Wilk Test and Kolmogorov-Smirnov Test were all less than 0.05, implying that the data significantly deviated from a normal distribution.
The NVP concentration medians for hair and plasma samples were 36.8ng/mL for 19.3ng/mL, respectively. In 25% of participants, the NVP plasma and hair concentrations were less than or equal to 17.1ng/mL and 5.2ng/mL, respectively. Additionally, in 75% of participants, NVP hair and plasma concentrations were less than or equal to 102.3ng/mL and 562.6ng/mL respectively. The hair samples' interquartile range (IQR) was (17.10 to 102.30ng/mL), which means that 50% of the hair NVP concentrations were between 17.10 and 102.30ng/mL. The interquartile range (IQR) for the plasma samples were (5.23 to 562.65ng/mL). This also means that 50% of the plasma NVP concentrations were between 5.23 and 562.65ng/mL.

The hair NVP concentration ranged between 5.4 to 1211.5ng/mL while the plasma (Figure 1). NVP concentrations ranged between 0 to 5000ng/mL. The plasma concentration was greatly dispersed as compared to the hair concentration. This was in line with the report by [8] that high upper concentration limits of NVP's plasma concentration extended up to 9000ng/mL. Low plasma NVP concentrations can arise due to poor adherence.[12] However, some patients can still have low plasma levels of NVP, even while still adhering to the ART regimen. This

could result from some factors such as interpatient variability in exposure to NVP, drug interactions, and interaction of drug with food.[12]

The variance of the NVP levels in the hair and plasma samples were 14793.4 and 197158.0, respectively. There was a high variability of NVP levels amongst the participants in hair as compared to plasma samples. The high variability of NVP levels in plasma across could arise due to differences in patients' body mass index, age, race, rate of metabolism, and genetic.[7,13]

Comparison of NVP levels in the plasma and matched hair samples

The hair and matched plasma samples were analysed and compared to determine whether there is a significant difference in the levels of NVP between the two samples. The representative chromatograms of hair and matched plasma samples are as shown in (Figure 2). The mean NVP concentration in hair was 104.9ng/mL, while the mean NVP concentration in matched plasma samples was 525.4ng/mL. Wilcoxon signed-rank test was used to test the hypothesis and revealed that there was no significant difference between the level of NVP in hair and matched plasma samples (Table 3). These results matched what Gandhi's and others found $P > 0.05$: a significant difference between efavirenz concentrations in the hair and plasma samples.[14,15]

Nevirapine and efavirenz are two of the most commonly prescribed NNRTIs. The WHO recommends regimens consisting of a NNRTI backbone with either efavirenz or nevirapine.[16] This showed that the drug deposition in the hair depends on its concentration in the plasma and thus concluded that NVP concentrations in hair samples are, therefore, a strong measure of adherence just like the plasma samples.[17]

FIGURE 1: Representative LC-MS/MS chromatogram of hair sample showing the compounds's and their precursor ions, and product ions are 267>226 and 237>197 for NVP and CBZ respectively

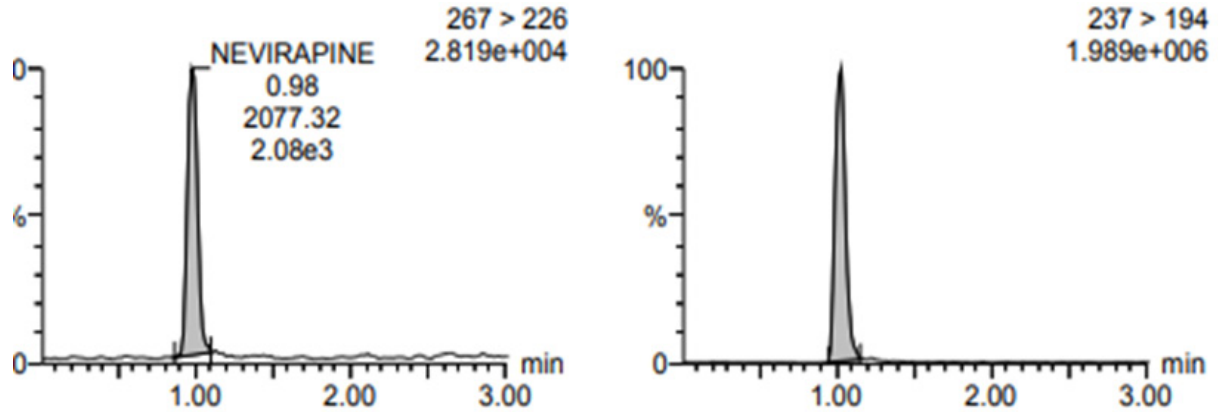

FIGURE 2: Representative LC-MS/MS chromatogram of matched plasma sample showing NVP precursor ions, and product ions, 267>226 respectively

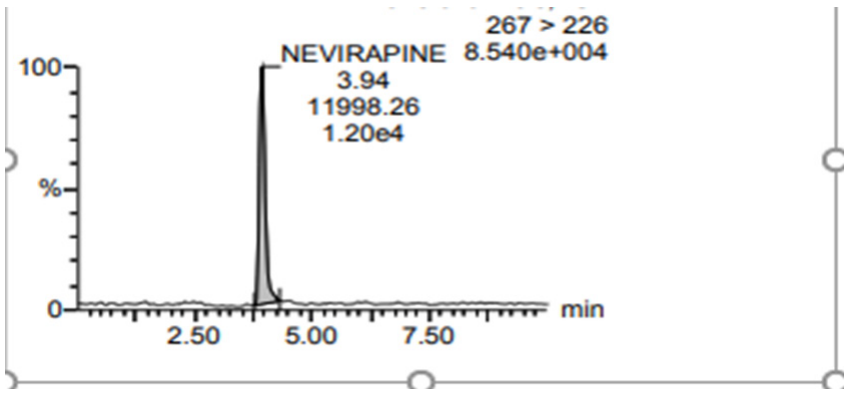

TABLE 1: Demographic Characteristics of Study Participants (N=413)

Variable		Frequency	Percentage
Age (years)	Median (range)	41 (34-49)	
	20-30	69	16.5
	31-40	135	32.6
	41-50	128	30.8
	>51	81	20.1
Gender	Female	242	58.4
	Male	171	41.6
Viral load	>1000copies/mL	210	50.8
	<1000copies/mL	47	11.2
samples types	Plasma samples	328	74.6
	Hair samples	223	25.4
Donated samples	Matched hair and plasma samples	94	22.0
	Donated only one sample type	329	78.0
Hair samples >10mg	Yes	105	47.1
	No	118	52.9
Plasma samples in good-conditions for analysis	Yes	308	93.9
	No	20	6.1
Duration on treatment	Median (IQR)	6	(3 - 11)
	1 - 5 Years	201	48.5
	5 - 10 Years	104	25.1
	>11 Years	108	26.4
Initial ARV type	lamivudine, nevirapine, stavudine	102	24.5
	lamivudine, efavirenz, tenofovir	194	46.8
	lamivudine, efavirenz, zidovudine	32	7.7
	lamivudine, nevirapine, tenofovir	26	6.3
	lamivudine, nevirapine, zidovudine	54	14.6
Changed initial ARV type	Yes	187	45.3
	No	226	54.7
Current ARV type	lamivudine, nevirapine, tenofovir	340	82.2
	lamivudine, nevirapine, zidovudine	72	17.3
	lamivudine, nevirapine, stavudine	1	0.5
Did not take ARV for a day or more	Yes	224	54.1
	No	189	45.9

TABLE 2: Mean, Median and Inter Quartile Range (IQR 25-75) Concentration of Nevirapine in Hair and Plasma Samples

Sample type	N	Mean (ng/mL)	Median (ng/mL)	IQR range (ng/mL)
Hair samples	105	106.1	36.8	17.1 – 102.3
Plasma samples	308	430.2	19.3	5.2 – 62.6

TABLE 3: Mean Concentration of Nevirapine in the Matched Hair and Plasma Samples

	N	Mean ± SE (ng/mL)
Hair concentration	94	104.90 ± 19.7
Plasma concentration	94	525.35 ± 96.2
Z = -0.928, *P>.05*		

CONCLUSION

There was no significant difference between the levels of NVP in hair and matched plasma samples. Therefore, as alternative to conventional plasma sample, hair sample can be used to monitor ART adherence, especially in our settings where laboratory facilities and skilled personnel to do phlebotomy are few or lacking. Determination of levels of other types of ARV drugs in hair and blood matched samples is highly recommended to ascertain whether hair sample can be used to monitor adherence to such drugs.

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