Evaluation of Saliva as an Alternative Matrix for Monitoring Plasma Zidovudine, Lamivudine and Nevirapine Concentrations in Rwanda[§]

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Abstract: Saliva may provide interesting advantages as matrix for compliance measurements, pharmacokinetic studies and therapeutic drug monitoring in resource limited countries. We investigated the feasibility of using saliva for compliance monitoring of zidovudine (ZDV), lamivudine (3TC) and nevirapine (NVP) in 29 HIV-1 infected patients from Rwanda. ZDV, 3TC and NVP drug levels were quantified by an LC/MS-MS method in plasma and stimulated saliva samples and compared using Bland-Altman analysis. Seven patients demonstrated undetectable saliva ZDV levels while five out of these seven also showed no 3TC salivary concentrations. For the other samples, we observed a good agreement between salivary and plasma concentrations of each antiretroviral drug. A significant relation between the difference in saliva and plasma ZDV concentrations and the average ZDV concentration in the two matrices was deduced as follows: $y = -380.15 + 1.79 x$. The log saliva and plasma concentration difference of both 3TC and NVP was consistent across the range of average log concentration. Overall, we showed large agreement limits suggesting a wide inter patient variability that may result in non-reliable plasma level predictions from saliva drug measurements. Therefore, our results indicate that saliva may serve as a valuable tool only for NVP compliance testing because of its high salivary concentration.

Keywords: Developing countries, HAART, lamivudine, nevirapine, patient compliance, saliva, zidovudine.

INTRODUCTION

Oral fluids have been introduced since the early eighties as a matrix for detection and quantification of drugs and drugs of abuse. Compared to blood sampling, whole saliva has several important advantages: fluid collection is non-invasive, skin irritation or bruising in patients with poor venous access can be avoided and sample collection is safe. Further, saliva is a cost-effective tool for screening a large population and allows multiple sample collections at any time of the day [1]. However, saliva cannot be used as a simple substitute for blood testing since pharmacokinetic characteristics of many drugs are more complex in saliva than in blood [2].

Few studies investigated the use of saliva for therapeutic drug monitoring (TDM) and/or compliance control studies in HIV infected patients undergoing antiretroviral therapy (HAART) [3-6]. Rolinski and collaborators have shown a good correlation between plasma and salivary concentrations of zidovudine (ZDV) in ten HIV-infected patients suggesting that stimulated saliva might be an appropriate specimen for ZDV-TDM [3]. Plasma nevirapine (NVP) concentrations were strongly correlated with stimulated saliva levels from NVP-treated adults [4]. Although indinavir concentrations in saliva were shown to be related to plasma concentrations, the authors of the study did not recommend saliva for TDM because of significant intra and inter individual variations [5]. However, a correlation between two matrix values does not automatically imply a good agreement between two methods of measurement. The Bland and Altman method [7] is the method of choice to assess graphically agreement between two body fluids. Using this method, agreement between plasma and non stimulated NVP saliva levels in children on multi-HAART regimen was reported [6].

The majority of HIV-infected individuals are living in Sub-Saharan Africa. More than 75 000 individuals received HAART in Rwanda in 2009. Although virological and immunological outcomes to HAART in Rwanda were comparable to western countries [8-10], routine supervision and adherence data are limited [10]. The aim of our study was to investigate the agreement between salivary and plasma concentrations of ZDV, lamivudine (3TC) and NVP for adherence and pharmacokinetic studies in resource limited settings.

MATERIAL AND METHODS

Study Group

The study was a cross-sectional design in a prospective cohort initiated by the ESTHER ("Ensemble de Solidarité Thérapeutique Hospitalière en Réseau") project at the HIV clinic of the Treatment Research AIDS Center (TRAC) in Kigali. All participants signed a written consent form before

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being enrolled into the survey. HAART treatment consisted of ZDV/3TC (Combivir®, GlaxoSmithKline, London) and NVP (Viramune®, Boehringer Ingelheim, Ingelheim) taken twice daily. The total daily dose was 600 mg ZDV, 300 mg 3TC and 400 mg NVP. Adherence to the antiretroviral (ARV) treatment was controlled by frequent questionnaires and pill counts. Patient characteristics are summarized in Table **1**.

Table 1.   Characteristics of the 29 HIV-1 Infected Patients

Patient Characteristics		Value
Gender, *n*	Women	23
	Men	6
Age, years	Mean (SD)	40.7 (8.1)
	Range	27 – 60
Body weight, kg	Mean (SD)	57.1 (9.1)
	Range	40 – 75
CD4 cell count[a], cells/mm³	Mean (SD)	195.0 (122.4)
	Range	3 – 588
WHO clinical stage, *n* (%)	1	1 (3)
	2	4 (14)
	3	19 (66)
	4	5 (17)

[a]CD4 cell counts were measured using a FACScalibur™ Flow Cytometer (Becton Dickinson, Franklin Lakes, USA); n, number of patients; SD, standard deviation; WHO, World Health Organization.

Sample Collection and Preparation

Blood and saliva samples were collected during a routine consultation, 3 to 5 hours (mean 225 min) after the last drug intake. Saliva was collected using a Salivette® (Sarstedt AG & Co., Nümbrecht, Germany) containing a roll-shaped saliva collector impregnated with citric acid to stimulate salivation. The swab was chewed for one to two minutes and placed back in the insert. Plasma and salivettes were shipped on dry ice to Luxembourg and stored at -20°C before analysis. Then the salivettes were centrifuged for 5 minutes at 3 500 g to collect the saliva while the swabs were washed with an ethyl acetate/methanol mixture (1/1, v/v). Extracts were evaporated and the residual saliva added to the previously collected saliva. One milliliter of saliva or plasma was then mixed to 1 ml ammonium buffer at pH 9.5 and Mebeverine which served as internal standard. Unbound ARVs were extracted with solid phase extraction cartridges as described earlier [11, 12]. After evaporating the extracts to dryness under nitrogen at 40°C, the residues were reconstituted in 100 μl ammonium formate buffer (pH 3.8) for quantitative analysis.

Antiretroviral Drug Concentration Measurements

Detection and quantification of ARV drugs was carried out on a high performance liquid chromatography system coupled to a LCQ™ Duo Ion Trap Detector (Thermo Electron, Zellik, Belgium) equipped with an electrospray ionization interface and operated in both positive and negative ion mode. The separation of sample components

was achieved on an XTerra® MS C18 column (5 μm particle size, 3.9 x 150 mm, Waters, Overijse, Belgium), equipped with a similar pre-column (10 mm). The mobile phase consisted of a mixture of 5 mM ammonium formate buffer at pH 3.8 and acetonitrile. Validation parameters for the quantification method included determination of extraction yield, establish interday and intraday accuracy and repeatability as well as verification of absence of ion suppression in the calibration range. Six point calibration curves covering the therapeutic ranges of the ARV drugs were established and all r^2 were ≥ 0.98.

Statistics

ARV concentrations were analyzed using R v.2.10.1. Applying the Bland-Altman method [13], log differences between saliva and plasma measurements were plotted against their average log concentration to determine the bias and the 95% limits of agreement (bias ± 1.96 standard deviation, SD). The Shapiro-Wilk test was used to assess normality of log differences, one condition of the Bland-Altman method. A linear regression analysis was used to confirm the lack of relationship between the two measurements and the average concentration (both expressed in log). If saliva minus plasma levels were still related to the average concentration after log transformation, a regression approach for non-uniform differences was applied [14] and regression based limits of agreement for the difference in drug concentrations were calculated. According to the Bland and Altman's recommendation, 95% limits of agreement for a saliva/plasma (S/P) ratio with back-transformed log values was then calculated.

RESULTS

Mean and median concentrations of unbound ZDV, 3TC and NVP in saliva and plasma of the 29 HIV-1 infected Rwandan patients are given in Table **2**. Seven patients demonstrated saliva ZDV concentrations below the detection limit with two also showing undetectable plasma ZDV. Among these seven patients, five had indiscernible 3TC salivary levels. One patient was considered as an outlier for saliva ZDV concentrations because the range observed was 10 fold higher than the physiological concentrations. These saliva and plasma concentrations have not been included in the statistical analysis. NVP was detected in all plasma and saliva samples (concentrations ranging from 207 to 9 730 ng/ml in saliva and from 417 to 8 917 ng/ml in plasma).

Average unbound ZDV was higher in the saliva compartment than in the plasma with a mean S/P ratio of 4.0 ± 9.8 (mean ± SD) whereas unbound 3TC plasma concentrations were higher than salivary concentrations in all patients (mean S/P ratio = 0.3 ± 0.2, Table **2**). Mean concentrations of unbound NVP were similar in plasma and in saliva with a mean S/P ratio of 1.5 ± 2.1. For NVP, 15/29 patients had a S/P ratio < 1 indicating a high heterogeneity of the blood-saliva diffusion between individuals. Interestingly, most patients (10/15) with a ratio < 1 for NVP had also a ratio < 1 for ZDV.

A relationship between the differences of plasma and saliva drug concentrations and the average concentrations in the two measurements was established for each drug

Table 2.   **Mean and Median of Antiretroviral Drug Concentrations in Plasma and Saliva**

	Zidovudine	Lamivudine	Nevirapine
Plasma concentrations[a] **Mean (SD)** **Median (IQR)**	*n* = 26 205 (179) 148 (107 - 286)	*n* = 29 1 349 (759) 1 213 (837 - 1 965)	*n* = 29 3 045 (2 302) 2 707 (914 - 4 586)
Salivary concentrations[b] **Mean (SD)** **Median (IQR)**	*n* = 21 498 (789) 238 (84 - 612)	*n* = 24 372 (438) 197 (161 - 350)	*n* = 29 2 769 (2 513) 1 558 (462 - 4 547)
Saliva/Plasma ratio **Mean (SD)**	*n* = 21 4.0 (9.8)	*n* = 24 0.3 (0.2)	*n* = 29 1.5 (2.1)

[a]Unbound plasma concentrations in ng/ml; [b]Unbound saliva concentrations in ng/ml; IQR, interquartile range; n, number of patients; SD, standard deviation.

(p<0.05; Table **3**). Therefore, each sample was finally defined by the log saliva (S) – log plasma (P) difference and the average log concentrations [7, 13, 14]. The log-transformed measurement differences followed the normal distribution for each drug. The ZDV plot revealed a linear relationship between the log difference and average log concentration whereas the log difference of 3TC and NVP were consistent across the range of average log concentrations (Fig. **1**). The log-based linear regression model confirmed the independence of the log(S)-log(P) differences and log average for both 3TC and NVP (Table **3**). The plots revealed a good agreement between salivary and plasma concentrations with only one sample outside the limits of agreement for NVP (Fig. **1**). However, the estimated limits of agreement between the two matrices were relatively high for both 3TC and NVP (within 0.03 and 1.13 for the 3TC S/P ratio and 0.06 and 9.09 for the NVP S/P ratio). Using linear regression analyses, we could confirm a significant relationship between the log(S) – log(P) differences and the average ZDV log concentration (p< 0.05, Table **3**). Therefore, regression based limits of agreement for non-uniform differences in ZDV concentrations were calculated as previously described [14]. As shown in Fig. (**2**), the differences in ZDV saliva and plasma concentrations increased with increasing drug concentrations. The statistical relation between the ZDV difference saliva – plasma (y) and the mean concentrations (x) was expressed as follows: y = -380.15 + 1.79 x. A good agreement between the two values was observed; only one ZDV sample was outside the limits of agreement but large 95% limits of agreement were obtained (-1 104.84 + 1.79 x, 344.54 + 1.79 x).

DISCUSSION

A number of clinical trials have demonstrated that plasma concentrations of antiretroviral drugs are an important factor for treatment response [15]. Concentration based antiretroviral drug dose reduction might increase access to safer therapy while preserving viral load suppression [16, 17]. In contrast, inadequate drug concentrations, often due to drug adherence failure, may lead to appearance of toxicity or drug resistance mutations. Lack of compliance of HAART-treated patients is estimated to occur between 40% and 60% of patients [18]. Combined with resistance tests, assessment of adherence may prove to be useful in developing countries [19] to preserve limited

second line treatment. Genotypic resistance testing has been initiated in Rwanda [8, 9, 20] and close monitoring of patients at risk has been proposed as a relevant strategy [21]. To our knowledge, our study is the first study evaluating the utility of saliva in a Sub-Saharan population for which specific genetic background, medications and diet might influence differently antiretroviral pharmacokinetics both in plasma and saliva.

Although we showed a good agreement between saliva and plasma levels of ZDV, 3TC and NVP, the high inter individual variability represented by large 95% limits of agreement does not allow an accurate quantitative deduction of plasma ARV levels based on saliva ARV concentrations. In plasma, the inter patient variability of antiretroviral drugs has been reported by us [11, 12] and by others [22, 23] and has been attributed to genetic influences, diet and/or presence of other drugs and diseases [24-26]. In saliva, the parameters responsible for drug concentration variations may be salivary flow rate, salivary pH, and drug pharmacokinetics. The salivary flow rate varies as a function of nutritional and emotional state, age, presence of diseases and/or presence of other drugs [27]. High inter individual but low intra individual variation of saliva pH has been described [28]. Variations in S/P ratios between individuals may also be linked to differences in bound and unbound drug concentrations in plasma. This variation was reported to be high for NVP [29], relatively low for ZDV [30] and to vary as a function of age for 3TC [31].

Predicting plasma ARV concentrations from saliva is based on the assumption that saliva drug concentrations are related to unbound blood levels of the drug. The blood – saliva transfer of drugs appears to be a passive diffusion process. The S/P ratio has been described as a function of drug pKa, of differences in drug protein binding in blood and saliva and the pH in the saliva and plasma [32]. In normal physiological conditions (blood pH of 7.4 and a saliva pH range from 5.5 – 7.9), considering a pKa of 9.7 and an unbound drug fraction of 0.81 for ZDV, 4.3 and 0.64 for 3TC and 2.8 and 32 for NVP respectively, the S/P ratios for the three drugs were estimated to vary from 0.3 to 64 for ZDV, 0.1 to 2.0 for 3TC and 0.1 to 1.0 for NVP depending on the saliva pH. In our study, the average S/P values of unbound ZDV, 3TC and NVP were 4.0, 0.3 and 1.5 respectively. With its pKa of 9.7, ZDV is present in plasma in a non-ionized form and has fewer difficulties to diffuse
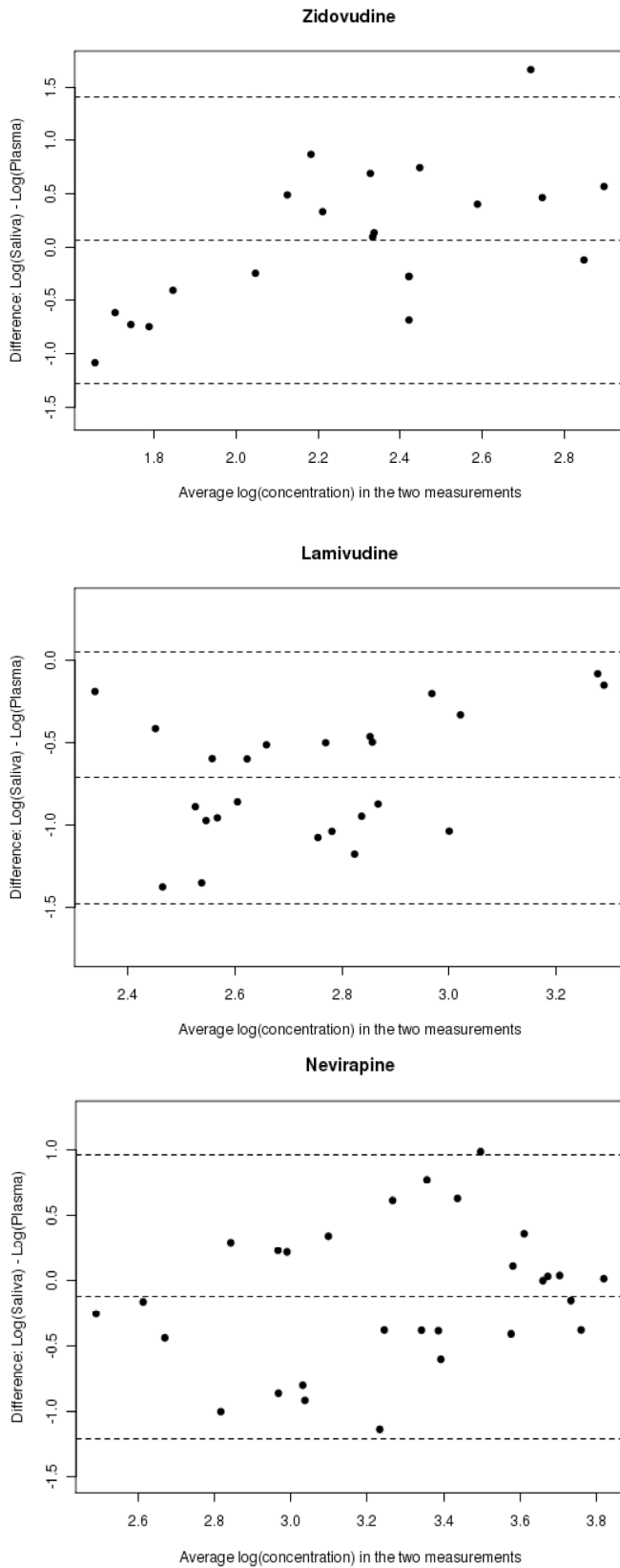
Zidovudine

Lamivudine

Nevirapine

Fig. (1). Zidovudine (ZDV), lamivudine (3TC) and nevirapine (NVP) Bland-Altman plots representing the differences between log saliva and log plasma as a function of the average log concentrations. The ZDV plot showed a relationship between the log differences and the mean log concentrations. A good agreement between the two body fluids for 3TC and NVP was observed.
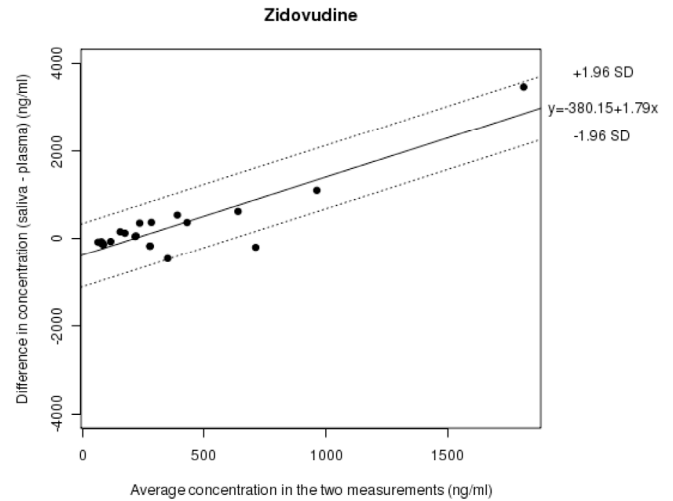
Zidovudine

Fig. (2). Linear regression plot between the saliva minus plasma concentration differences and the average concentrations in the two matrices following the equation y = -380.15 + 1.79 x. One sample was outside the limits of agreement.

Table 3. **Statistical p-Values of the Linear Regression Analysis for Zidovudine, Lamivudine and Nevirapine Bland-Altman Plots**

	Zidovudine	**Lamivudine**	**Nevirapine**
p-value[a]	8.62 E-10	0.02	0.04
p-value[b]	0.00224	0.055	0.171

[a]Relation between saliva minus plasma difference and average concentrations;
[b]Relation between difference of log saliva and log plasma and average log concentrations.

through the cell membranes than the ionized 3TC (pKa of 4.3). Thus, unbound 3TC concentrations were found to be higher in plasma than in saliva. Independent of the presence of ionized NVP molecules at pH 7.4, the mean unbound salivary concentration of NVP was as high as the plasma levels that might be a consequence of its greater lipophilicity as compared to ZDV and 3TC.

Based on correlation coefficients, ZDV and NVP concentrations were previously significantly related in the two matrices [3, 4, 6]. We also observed a significant correlation by the non parametric Spearman correlation rank test for each ARV (data not shown). However, the use of correlation coefficients to compare two different biological compartments is misleading [7, 13, 14] as the physiological parameters modulating the drug concentrations in the two body fluids are diverse. Using the Bland-Altman analysis, the 3TC and NVP plots indicated a non equal distribution in the two matrices among the patients, while the ZDV plot revealed a linear relationship between the log difference in saliva and plasma concentrations and the mean log concentrations. Although an accurate prediction of ZDV plasma concentrations can be deduced from a linear equation, the large 95% limits of agreement as well as the 7 samples without detectable saliva concentrations have to be taken into account and may hint towards other influent parameters in these patients.

TDM is recommended to decipher drug interactions or to explain poor response to treatment and side-effects related to high dose of antiretroviral drugs in HIV-infected patients. Considering that we did not address the question of TDM in our study that only included a limited number of patients, the usability of saliva seems ambiguous. In conclusion, our data suggest that saliva may only be used for NVP as a valuable tool for compliance testing in resource limited countries.

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