Original Article

Exploring The Use Of A Mobile Microvolume Spectrophotometer For Saliva-Based Monitoring Of Antiretroviral Drugs

Zamrotul Izzah[a,b,c*], Frank Pierik[a], Leanne Ambrosio[d], Erwin M. Jongedijk[a], Christoffer Åberg[b], Daan J. Touw[a,b]

[a] Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
[b] Department of Pharmaceutical Analysis, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, the Netherlands
[c] Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
[d] Hospital pharmacist in training, Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht, the Netherlands

*Corresponding author: Zamrotul Izzah
Department of Clinical Pharmacy and Pharmacology
University Medical Center Groningen
University of Groningen
Hanzeplein 1
9713 GZ Groningen, the Netherlands
E-mail: z.izzah@rug.nl | zamrotulizzah@ff.unair.ac.id
DOI: 10.47750/pnr.2024.14.04.103

Abstract

Introduction: Therapeutic drug monitoring can be used to evaluate the efficacy of HIV treatment and identify non-adherence. This study explored the possibility of an easy-to-use and non-invasive assay for monitoring antiretroviral drugs in saliva. Theoretical salivary concentrations from previous studies were 0.4 – 25.8 ng/ml for tenofovir, 15 – 718 ng/ml for lamivudine, and 3.125 – 100 ng/ml for efavirenz.
Methods: A mobile microvolume UV/visible light spectrophotometer operating at wavelengths of 200 – 900 nm was used. Drug-free saliva samples were obtained from six healthy volunteers and were spiked with antiretroviral drug.
Results: Calibration curves were made over a range of 2,500 – 50,000 ng/ml for tenofovir in ultrapure water (R^2 = 0.9994) at 261 nm, 200 – 800 ng/ml for lamivudine in saliva (R^2 = 0.7091) at 271 nm, and 1,500 – 4,000 ng/ml for efavirenz in saliva (R^2 = 0.9152) at 247 nm. The lowest limit of quantification (LLOQ) was determined to be 2,500 ng/ml for tenofovir, 200 ng/ml for lamivudine, and 2,000 ng/ml for efavirenz. The total absorbance of lamivudine and efavirenz in saliva exceeded the linear range for analysis.
Conclusions: The proposed spectrophotometer assay was not able to quantify tenofovir, lamivudine, and efavirenz in saliva. Further research with improved methods, different matrices or devices could be explored.

Keywords: alternative sampling; antiretroviral drug; drug monitoring; saliva; spectrophotometry

INTRODUCTION

The advent of antiretroviral therapy (ART) for the treatment of human immunodeficiency virus (HIV) infection has increased survival and improved clinical outcomes, turning the infection into a manageable chronic disease [1,2]. The World Health Organization (WHO) recommends initial ART regimens, which consist of a combination of two

nucleoside/nucleotide reverse transcriptase inhibitors and an integrase strand transfer inhibitor or a non-nucleoside reverse transcriptase inhibitor [3,4]. Tenofovir, lamivudine, and efavirenz are among the preferred first-line ART regimens to suppress HIV replication for adults and adolescents and these drugs are available as a fixed-dose combination in a single tablet regimen [3].

Optimal antiretroviral drug concentrations are an important factor for successful HIV viral suppression [2,5]. High plasma concentrations are associated with treatment efficacy, but increase the risk of toxicities [5]. In contrast, insufficient or undetectable drug concentrations due to low adherence to ART can lead to treatment failure and emergence of drug resistance [6,7]. Therefore, poor ART adherence can be suspected when a patient experiences a high detectable HIV viral load without any apparent reason. In practice, viral load is checked every 6 – 12 months, and thus treatment failure due to ART adherence problems is likely to be discovered late. This puts the patient at a high risk for developing severe infection and drug resistance.

Therapeutic drug monitoring (TDM) in HIV has been recommended for monitoring and evaluating treatment efficacy and short-term adherence to ART [2,8]. TDM can uncover current adherence problems, allowing for the identification of patients in need of adherence-enhancing interventions [8]. However, despite its importance, implementation of TDM in HIV endemic settings has logistic and financial challenges. Plasma has been used intensively for TDM, but venous blood sampling is invasive and can therefore be an inconvenience for patients [9]. Furthermore, liquid chromatography tandem mass spectrometry (LC-MS/MS), which has become the method-of-choice for the bioanalysis of antiretroviral drugs [10], requires costly laboratory procedures which hinder its application, particularly in resource-limited settings. Therefore, an easy-to-use, scalable, and affordable assay with a preferably non-invasive matrix to monitor ART treatment would be highly desirable.

Recently, saliva-based monitoring of antituberculosis drugs (levofloxacin and linezolid) has been developed and validated successfully using a mobile microvolume ultraviolet (UV)/visible light (Vis) spectrophotometer [11,12]. The assay requires a minimum of laboratory skills and was implemented successfully in a pharmacokinetic study of levofloxacin in African patients with multidrug resistant tuberculosis [13]. Furthermore, saliva has been used as an emerging, non-invasive, and patient-friendly matrix alternative to blood assay for TDM. Drug penetration into oral fluid is a prerequisite for the test to be useful [14].

Following the implementation in TB drugs, a saliva assay has the potential to be applied for other drugs, e.g. in HIV treatment. Tenofovir, lamivudine, and efavirenz are the backbone for HIV treatment in Indonesia and available as a fixed-dose combination tablet. Those three drugs have chromophore chains (**Figure S1**) and thus exhibit a UV spectrum. Detectable levels of those drugs in saliva of patients living with HIV have also been reported [15–17]. Theoretical salivary concentrations from previous studies were 0.4 – 25.8 ng/ml for tenofovir, 15 – 718 ng/ml for lamivudine, and 3 – 100 ng/ml for efavirenz. Against this backdrop, this article describes our attempts in exploring the potential of using a mobile microvolume spectrophotometer for quantification of tenofovir, lamivudine, and efavirenz in human saliva to enable objective monitoring of ART use in resource-limited HIV endemic settings.

MATERIALS AND METHODS

Chemicals

Tenofovir and lamivudine (purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Efavirenz (purity > 99%) was kindly supplied by the Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT, The Hague, the Netherlands). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Millipore Corporation, MA, USA). Hydrochloric acid and sodium hydroxide concentrates were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Naphthol (β-naphthol) was obtained from Flucka Chemicals (Buches, Switzerland).

Hydrochloric acid 0.1 M solution was prepared by diluting 8.3 ml of concentrated hydrochloric acid in ultrapure water to a total volume of 1.0 l. Sodium hydroxide 4.0 M solution was prepared by dissolving 56.0 g sodium hydroxide concentrate in ultrapure water for a total volume of 350.0 ml. β-naphthol solution was prepared from 0.5 g β-naphthol dissolved in 4.0 ml sodium hydroxide (8.5% w/w) and diluted in ultrapure water to a total volume of 10.0 ml. Molybdovanadate reagent was prepared by from 4.0 g ammonium molybdate and 0.1 g ammonium vanadate

dissolved in 70.0 ml of ultrapure water. The solution was mixed with 20.0 ml of nitric acid (63.0 – 70.0% w/w) and then diluted with ultrapure water to get a final volume of 100.0 ml.

Instrument

Samples were measured on a mobile microvolume UV/Vis spectrophotometer, NanoPhotometer NP80® (Implen, München, Germany), with a wavelength range of 200 – 900 nm, and a scan time of 2.5 – 4 s. A pathlength of 0.67 mm was used when the sample was placed on the drop tray, and a pathlength of 10 mm was used when the sample was in a cuvette. The baseline correction and smoothing filter were switched off. Ultrapure water was used as a blank. Samples were placed on the drop tray using a disposable Pasteur pipette (≥ 3 µl for tenofovir) or a cuvette (100 µl for lamivudine and efavirenz). The surface of the drop tray was cleaned with deionized water and 70% ethanol and dried using lint-free tissues after each measurement.

Stock solution preparation

Stock solutions were made by dissolving tenofovir and lamivudine in ultrapure water. Efavirenz was first dissolved in 50% (v/v) methanol/water before further dilution in ultrapure water. The total volume of diluted stock solution that was added to the blank saliva was kept at 5.0% (v/v).

Sample preparation

Blank saliva was collected from six healthy individuals using Salivette® (Sarstedt, Nümbrecht, Germany) after rinsing their mouth with water. Volunteers were individually approached and recruited; they were between 23 and 26 years old, used no medications except oral contraceptives for females, and provided their written consent prior to saliva collection. In order to collect saliva, the cotton swab was removed from the Salivette® tube and placed in the mouth. After being chewed for 2 min, the saliva-soaked swab was returned to the Salivette® tube and then closed with the plug. Following saliva collection, the Salivette® tubes were centrifuged at 2,997 rpm for five minutes using a Rotina 420 Centrifuge (Hettich Benelux, Geldermalsen, the Netherlands) and filtered through a Millex-GP filter (Merck Millipore, Carrigtwohill, Ireland) with a pore size of 0.22 µm using a 5.0 ml syringe.

Since absorbance of tenofovir was too low for our objective, different strategies were explored to increase the absorbance. Each experiment was performed at least in triplicate. First, the use of different pH solutions was done by adding 15 drops of 0.1 M hydrochloric acid solution (final pH of 2.5) or nine drops of 4.0 M sodium hydroxide solution (final pH of 12.0) to 15.0 ml of tenofovir solution (50,000 ng/ml). Results were compared with tenofovir solution without any additives (pH 7.0). Second, the initiation of a reaction between two potentially reactive groups of tenofovir and indicators was performed to increase the absorbance of the solution. The primary amine group of tenofovir was reacted with a freshly prepared β-naphthol solution as follows: 2.0 ml of tenofovir solution was acidified with two drops of hydrochloric acid (73 mg/ml), after which 0.2 ml of sodium nitrite solution (100 mg/ml) was added and mixed. After two minutes, 1.0 ml of β-naphthol solution was added to the test solution and the solution was mixed before being measured with the mobile microvolume spectrophotometer. Alternatively, another reaction was performed with the phosphate group of tenofovir by adding 0.5 ml molybdovanadate reagent to 1.0 ml of tenofovir solution. The test solution was mixed and then left to react for five minutes before measurement.

Assay procedure

The spectrophotometry assay was explored according to European Medicines Agency (EMA) and Food and Drug Administration (FDA) guidelines [18,19] for its calibration curve, sensitivity, and selectivity.

The calibration standards of tenofovir consisted of concentrations at a range of 10 – 50,000 ng/ml. The lamivudine calibration standards consisted of concentrations at a range of 10 – 800 ng/ml. The calibration standards of efavirenz consisted of concentrations at a range of 5 – 4,000 ng/ml. These concentrations were based on the concentration ranges reported in saliva of patients with HIV [15–17]. A calibration curve was determined by plotting absorbance at the selected wavelength against concentration and performing linear regression, using at least six

calibration concentration levels. Each calibration standard was analysed at least in triplicate.

EMA/FDA guidelines require that back calculated concentrations of the calibration standards should be within ± 15% of the nominal concentrations, except at the lower limit of quantification (LLOQ) where the back calculated concentration should be within ± 20% of the nominal concentration [18,19]. Those criteria should be fulfilled by at least 75% of the calibration standards with a minimum of six concentration levels. If replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level. If a calibration standard does not comply with these criteria, the calibration curve without this standard should be re-evaluated, including regression analysis.

The assay sensitivity was based on the determination of the LLOQ using the lowest calibration standard in ultrapure water and calculated based on the analyte signal. The analyte response of the LLOQ was at least 5 times the response of a blank sample (ultrapure water) with the accuracy should be within ±20% of nominal concentrations and the precision (coefficient of variation) should not exceed 20% [18,19]. For selectivity, drug-free saliva samples obtained from six healthy volunteers were evaluated for interference. The guidelines require that measurements of these samples should be free of interference and result in responses less than 20% of the LLOQ [18,19].

All data calculations and analyses were performed by importing raw data into proprietary spreadsheets of Microsoft® Excel® for Microsoft 365 MSO (version 2210). Data are presented in frequencies, percentages, and mean (± standard deviation, SD) if applicable. The linearity of calibration curves was evaluated by the least square regression method using unweighted data. Results of assay are presented in charts and tables as appropriate.

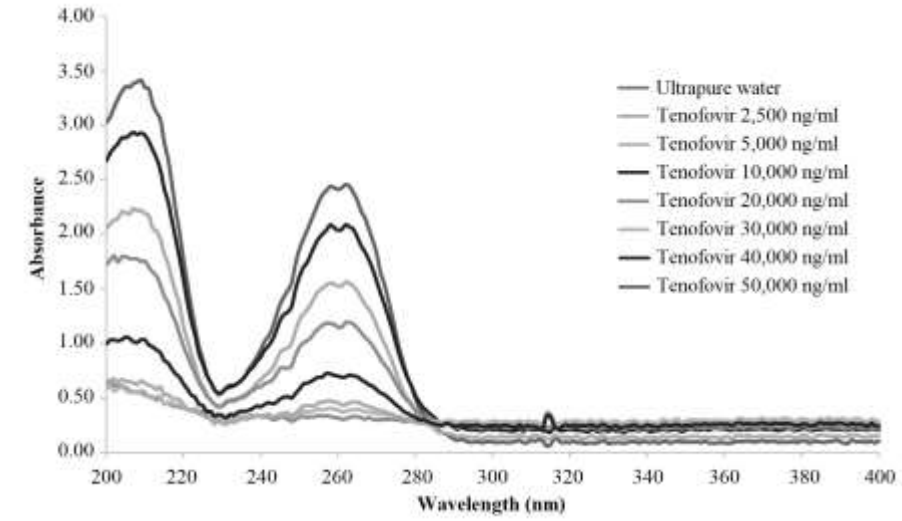
RESULTS

Tenofovir

The calibration standards of tenofovir in ultrapure water were measured at concentrations of 10, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 20,000, 30,000, 40,000, and 50,000 ng/ml. The maximum absorbance was observed at a wavelength of 261 nm. However, after evaluating those calibrations standards based on EMA/FDA guidelines, concentrations below 2,500 ng/ml were rejected first because the back calculated concentrations did not comply with the criteria (within ±15% or ±20% for LLOQ).

The calibration standards of tenofovir 2,500 – 50,000 ng/ml in ultrapure water were re-evaluated, including the regression analysis, and the results are shown in **Figure 1**. The spectra of tenofovir in ultrapure water showed clear baseline shifts and increasing magnitudes of absorbance at the 261 nm maximum. Linearity of the calibration curve evaluated at 261 nm was observed (R^2 = 0.9994) for tenofovir from 2,500 to 50,000 ng/ml in ultrapure water. The LLOQ in ultrapure water was determined to be 2,500 ng/ml. All the back calculated concentrations of tenofovir 2,500 – 50,000 ng/ml in ultrapure water deviated less than ±20% for the LLOQ and less than ±15% from the nominal concentrations (**Table S1**).

(A)



(B)

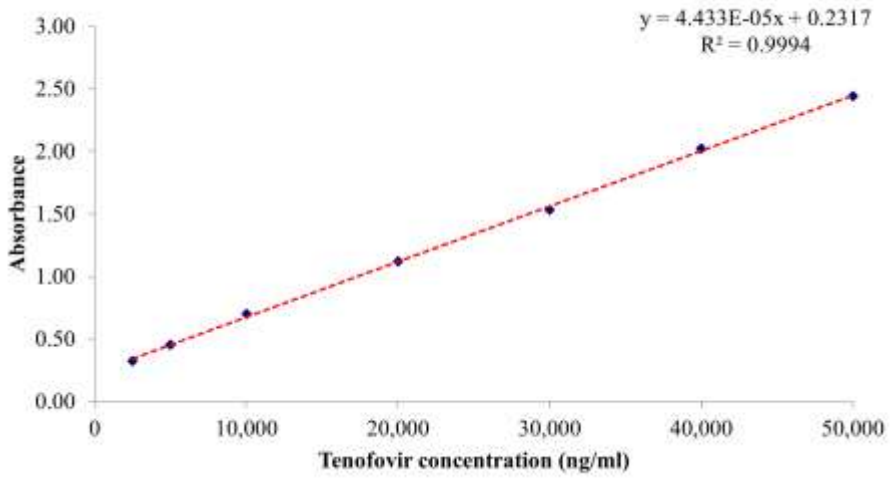


Figure 1: Spectra and absorbance of tenofovir in ultrapure water at concentrations 2,500 to 50,000 ng/ml. A: Spectra of tenofovir in ultrapure water at 200 – 400 nm; B: a calibration curve with a red-dashed trendline of tenofovir, evaluating the absorbance at 261 nm.

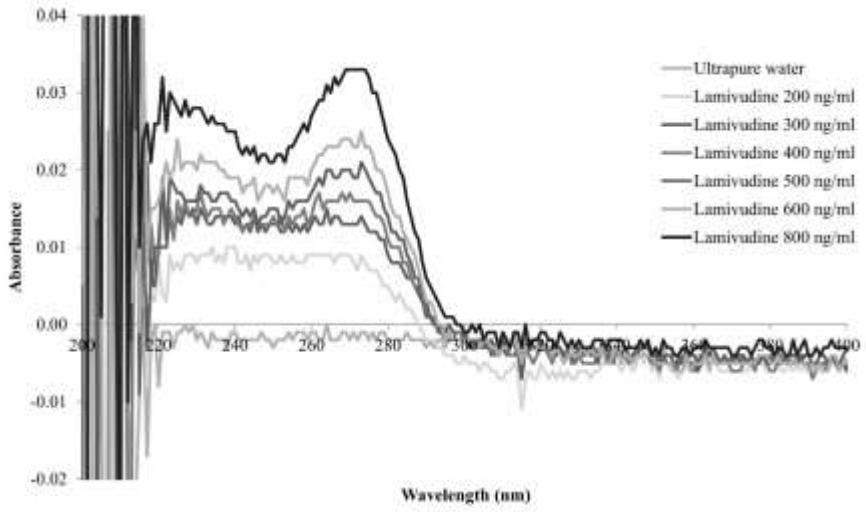
Since the LLOQ is higher than the lower tenofovir concentration reported in patients' saliva, several attempts were performed to lower the LLOQ before working on the saliva samples. Acidification of tenofovir solution 50,000 ng/ml decreased the absorbance at 261 nm from 2.16 ± 0.03 (at pH 7.0) to 2.09 ± 0.02 (at pH 2.5). Alkalinization of the solution also lowered the absorbance at 261 nm from 2.16 ± 0.03 (at pH 7.0) to 2.10 ± 0.005 (at pH 12.0). Alkalinization also caused a red-hyperchromic shift of the peak at 207 nm (at pH 7.0) to 205 nm (at pH 12.0) with a sharp increase in absorbance as shown in **Figure S2**. Furthermore, the reaction of tenofovir solution with sodium nitrite and β-naphthol solutions as well as with the molybdovanadate reagent produced a yellow-orange colour and precipitation. There were no differences for tenofovir absorbance observed at 261 nm.

All of the above findings showed that the LLOQ and calibration curve concentrations of tenofovir are much higher than the target range concentrations of tenofovir detected in saliva of patients with HIV [15], and thus no attempt at quantifying tenofovir in saliva was performed.

Lamivudine

The calibration standards of lamivudine in ultrapure water were measured at concentrations of 10, 50, 100, 200, 300, 400, 500, 600, 700, and 800 ng/ml. The maximum absorbance was observed at a wavelength of 271 nm. However, after evaluating those calibrations standards based on EMA/FDA guidelines, concentrations lower than 200 ng/ml as well as 700 ng/ml were rejected first because the back calculated concentrations did not meet the predefined criteria (within ±15% or ±20% for LLOQ).

(A)



(B)

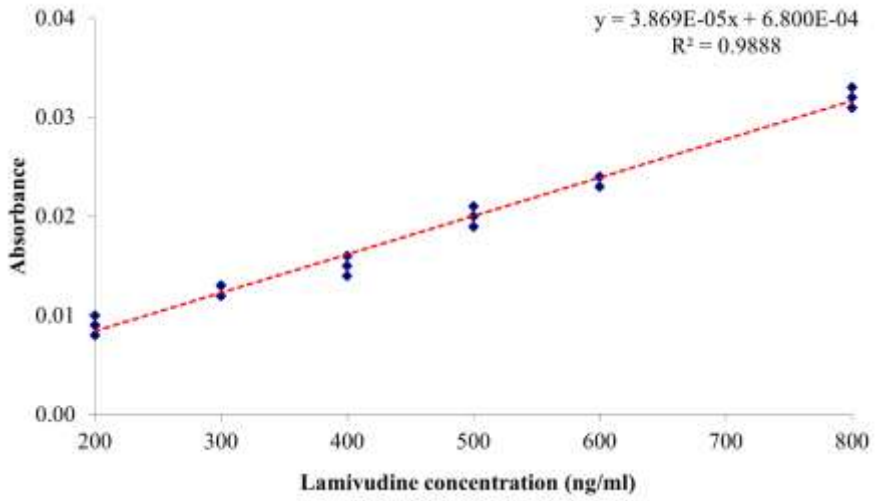


Figure 2: Spectra and absorbance of lamivudine in ultrapure water at concentrations 200 to 800 ng/ml. A: Spectra of lamivudine in ultrapure water at 200 – 400 nm; B: a calibration curve with a red-dashed trendline of lamivudine in ultrapure water, evaluating the absorbance at 271 nm.

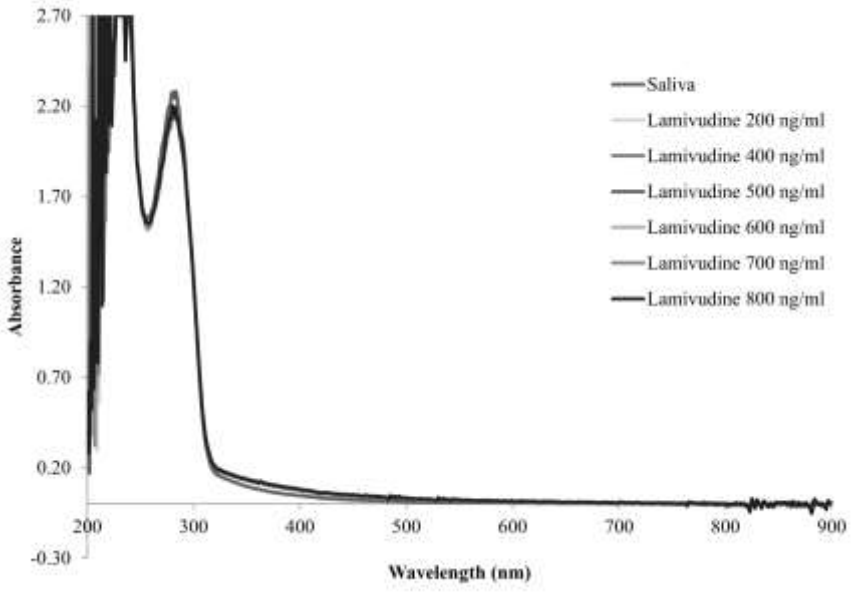
The calibration standards of lamivudine in ultrapure water at a range of 200 – 800 ng/ml as well as the regression analysis were re-evaluated, and the results are presented in **Figure 2**. The spectra of lamivudine in ultrapure water showed increasing magnitudes of the absorbance at the 271 nm maximum, and the calibration curve showed a linear trendline within concentrations that range from 200 to 800 ng/ml with an R^2 value of 0.9888. The LLOQ of

lamivudine in ultrapure water was determined to be 200 ng/ml. All the back calculated concentrations of lamivudine 200 – 800 ng/ml in ultrapure water deviated less than ±15% from the nominal concentrations and less than ±20% for the LLOQ (**Table S2**).

The calibration standards of lamivudine in saliva were also measured at concentrations of 10, 50, 100, 200, 300, 400, 500, 600, 700, and 800 ng/ml with the maximum absorbance was evaluated at a wavelength of 271 nm. However, after evaluating those calibrations standards based on EMA/FDA guidelines, concentrations lower than 200 ng/ml as well as 300 ng/ml were rejected first because the back calculated concentrations did not meet the predefined criteria (within ±15% or ±20% for LLOQ).

The calibration standards of lamivudine in saliva at a range of 200 – 800 ng/ml as well as the regression analysis were re-evaluated, and the results can be seen in **Figure 3**. The calibration curve of lamivudine for concentrations 200 – 800 ng/ml in saliva at 271 nm showed a low R^2 value (0.7091), and the absorbance does not vary much with concentration. However, even after the rejections, all but two of the remaining back calculated concentrations in saliva still differed more than 15% from the nominal concentrations and more than 20% for the LLOQ. The majority of the calibration standards tested per concentration level in the replicates did not fulfil the criteria (within ±15% or ±20% for LLOQ) (**Table S2**).

(A)



(B)

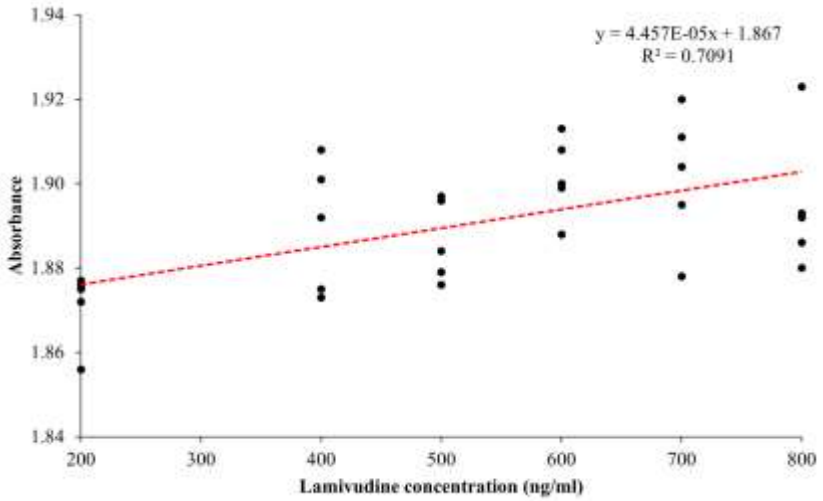


Figure 3: Spectra and absorbance of lamivudine in saliva at concentrations 200 to 800 ng/ml. A: Spectra of lamivudine in saliva at 200 – 900 nm; B: absorbance of lamivudine in saliva at a wavelength of 271 nm with a red-dashed trendline.

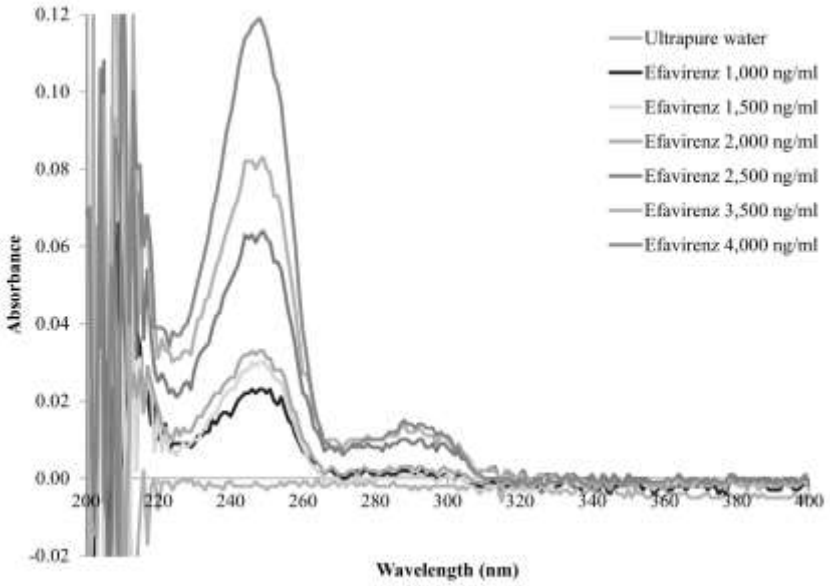
Six drug-free saliva samples from healthy individuals were further evaluated on their respective interference at a wavelength range of 240 – 300 nm. As can be seen in **Figure S3**, the absorbance of blank saliva is higher than that of the highest calibration standard of lamivudine at the wavelength of 271 nm.

Efavirenz

The calibration standards of efavirenz in ultrapure water were measured at concentrations of 5, 10, 50, 100, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, and 4,000 ng/ml. The maximum absorbance was observed at a wavelength of 247 nm. However, after evaluating those calibrations standards based on EMA/FDA guidelines, concentrations lower than 1,000 ng/ml as well as 3,000 ng/ml were rejected first because the back calculated concentrations did not meet the predefined criteria (within ±15% or ±20% for LLOQ).

The calibration standards of efavirenz in ultrapure water at a range of 1,000 – 4,000 ng/ml as well as the regression analysis were re-evaluated, and the results are presented in **Figure 4**. The spectra of efavirenz in ultrapure water showed increasing magnitudes of the absorbance at the 247 nm maximum. The calibration curve of efavirenz in ultrapure water with a concentration range of 1,000 to 4,000 ng/ml showed an R^2 value of 0.9309. The LLOQ of efavirenz in ultrapure water was determined to be 2,000 ng/ml. As can be seen in **Table S3**, efavirenz in ultrapure water at a concentration of 1,000 ng/ml should be rejected and this rejection resulted in less than six calibration standard levels fulfilled the FDA/EMA guidelines criteria (less than ±15% from the nominal concentrations and less than ±20% for the LLOQ) which were unsuitable for generating a new calibration curve.

(A)



(B)

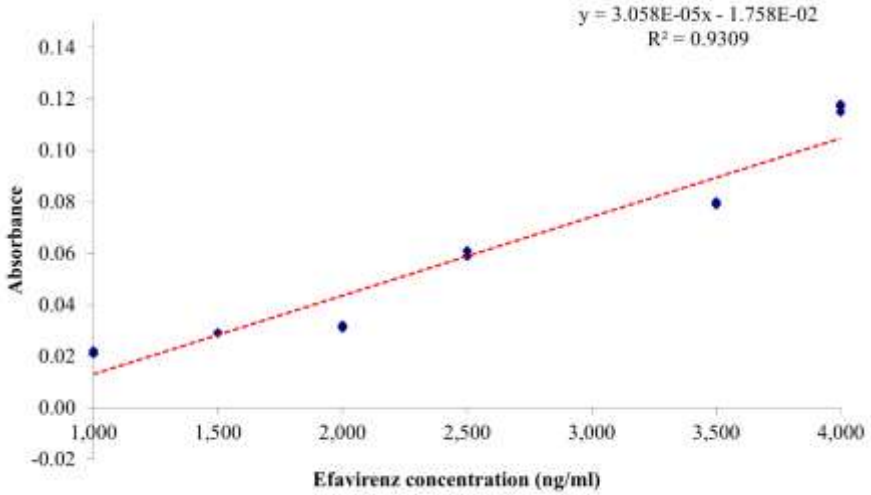
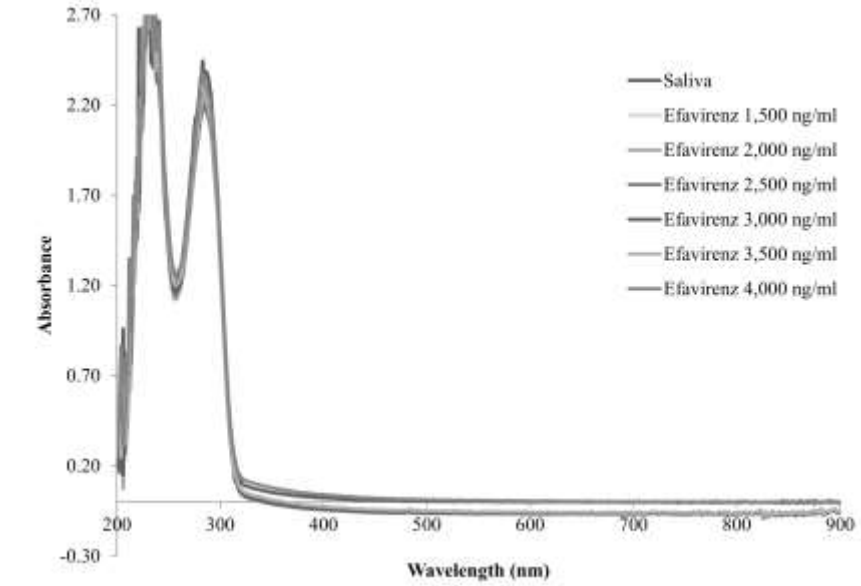


Figure 4: Spectra and absorbance of efavirenz in ultrapure water at concentrations 200 to 800 ng/ml. A: Spectra of efavirenz in ultrapure water at 200 – 400 nm; B: absorbance of efavirenz in ultrapure water with a red-dashed trendline, evaluating the absorbance at 247 nm.

(A)



(B)

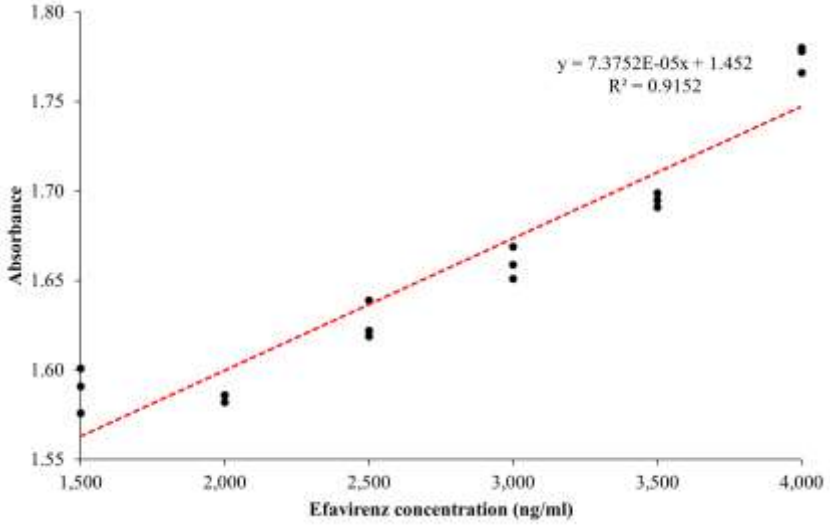


Figure 5: Spectra and absorbance of efavirenz in saliva at concentrations 1,500 to 4,000 ng/ml. A: Spectra of efavirenz in saliva at 200 – 900 nm; B: absorbance of efavirenz in saliva at a wavelength of 247 nm with a red-dashed trendline.

The calibration standards of efavirenz in saliva were also measured at concentrations of 5, 10, 50, 100, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, and 4,000 ng/ml with the maximum absorbance was evaluated at a wavelength of 247 nm. However, after evaluating those calibrations standards based on EMA/FDA guidelines, concentrations lower than 1,000 ng/ml were rejected first because the back calculated concentrations did not meet the predefined criteria (within ±15% or ±20% for LLOQ).

The calibration standards of efavirenz in saliva at a range of 1,500 – 4,000 ng/ml as well as the regression analysis were re-evaluated, and the results can be seen in **Figure 5**. The calibration curve in saliva at 247 nm showed an R^2 value of 0.9152. As can be seen in **Table S3**, efavirenz in saliva at a concentration of 1,500 ng/ml should be rejected and this rejection resulted in less than six calibration standard levels fulfilled the FDA/EMA guidelines criteria (less than ±15% from the nominal concentrations and less than ±20% for the LLOQ) which were unsuitable for generating a new calibration curve.

DISCUSSION

This is the first study exploring the use of a mobile microvolume UV/Vis spectrophotometer for quantification of antiretroviral drugs in saliva. However, the salivary assays of tenofovir, lamivudine, and efavirenz appeared not feasible due to the relatively high LLOQ for tenofovir and efavirenz and the matrix interference for efavirenz and lamivudine.

Tenofovir concentrations in saliva of patients living with HIV are 0.4 – 25.8 ng/ml (2 – 26 h post-dose) [15]. As the LLOQ for tenofovir in ultrapure water was 2,500 ng/ml, ~100 times higher than the target concentration, several strategies were explored to increase the absorbance of tenofovir and thereby to lower the LLOQ. Nevertheless, neither acidification nor alkalinization succeeded in increasing the absorbance. This could be explained based on the pKa of tenofovir (pKa = 3.74) [15]. The acidic group will be deprotonated in an alkaline solution with pH above 10.0 resulting in a net negative charge. The electrons can be distributed across a part of the molecule and affect light absorption. However, the effect is minimal due to the limited distribution across four atoms. In addition, a hyperchromic shift observed at 205 nm in the spectrum of the alkaline solution could not be used to quantify tenofovir, even though at this wavelength the absorbance was much higher compared to the absorbance at pH 2.5 and 7.0. Endogenous substances in saliva appear to absorb the light within that wavelength and thus may affect the selectivity of tenofovir [20]. Similarly, the LLOQ of efavirenz is also much higher than the efavirenz concentrations in saliva of patients with HIV at 3.125 to 100 ng/ml [17]. Thus, this proposed assay is unsuitable to quantify efavirenz in saliva.

The calibration curves of efavirenz and lamivudine in human saliva failed to meet the FDA and EMA requirements [18,19], and thus the calibration standards should be rejected from this assay. The possible source of the failure is that the total absorbance at the wavelength of maximum absorption exceeds the linear range of the spectrophotometer. The signal of blank saliva at 247 nm or 271 nm is higher than that of the highest calibration standard of efavirenz in ultrapure water (4,000 ng/ml) or lamivudine in ultrapure water (800 ng/ml). The signal of efavirenz or lamivudine might be suppressed in the salivary samples. Therefore, the assay seems not suitable to quantify lamivudine and efavirenz directly in saliva. Other strategies could be explored to improve the assay.

The Lambert-Beer's law states that the absorbance of light is directly proportional to the concentration of the absorbing components [21]. For a substance following this principle, zeroth-order spectra correlate with the concentration. However, the response measured at the wavelength of the maximum absorbance can be affected by endogenous compounds, such as proteins, present in saliva as can be seen from the lamivudine and efavirenz samples. Using a less sensitive wavelength (e.g. ~230 nm for lamivudine or ~280 nm for efavirenz) could be applied to decrease the total absorbance of saliva samples within the linear range of the detector. However, this approach was not feasible in the present study as the total absorbance at those wavelengths were still observed exceeding the linear range. The assay could be possibly improved in the future by exploring possible strategies to bring the total signal at the wavelength of maximum absorption into the linear range for analysis, e.g. by diluting the saliva samples in blank (ultrapure water), or shortening the pathlength with the use of the drop tray or different cuvette sizes [19,21]. Since the LLOQ of lamivudine in ultrapure water in the present study (200 ng/ml) is within the reported lamivudine concentrations in saliva of patients with HIV (15 – 718 ng/ml) [16], improving the assay might be promising to increase its applicability. Once all data are within the validated range for analysis and if the inter-patient variance of the blank matrix remains, derivative spectroscopy using the Savitzky-Golay filter may be applied. This may increase the resolution of the spectrum and decrease baseline shifts by filtering out the analyte signal from the total signal which, in turn, can increase the selectivity and specificity of the detection of analyte [11,22].

Given that the purposed salivary monitoring assay with the mobile microvolume spectrophotometer is not feasible for tenofovir, lamivudine, and efavirenz, other strategies using different matrices or more selective devices may also be further investigated. Low penetration of antiretroviral drugs into saliva due to their physicochemical properties, such as a low pKa value in the case of tenofovir, are a concern as the drugs cannot easily diffuse across the salivary gland [15,16]. Another non-invasive matrix such as urine could be explored. Tenofovir concentrations have been found to be ~100 times higher in urine than in saliva [15,23], and urinary tenofovir concentrations are strongly correlated with plasma [23].

The recent introduction of an enzyme immunoassay for a qualitative drug level testing of efavirenz, lopinavir, and dolutegravir in plasma [24] and tenofovir urine testing using dipstick technology [25,26] are advancements in detecting ART nonadherence. These assays are promising and can be further explored with the use of non-invasive matrices and other antiretroviral drugs in resource-limited settings. Furthermore, a more selective analytical method, such as a mobile high performance liquid chromatography (HPLC) system with UV/Vis detector could be investigated for an easy and affordable assay [27].

Of importance, this study has demonstrated that compounds with low concentrations in saliva and having the maximum absorbance within the range of wavelengths of endogenous compounds of blank saliva (240 – 300 nm) are difficult to be directly quantified in saliva by spectrophotometry. This might become a great obstacle for the monitoring of tenofovir, lamivudine, and efavirenz in saliva. The wavelength of maximum absorbance of these three drugs are relatively close to those of the blank saliva and the absorption peaks could overlap in the UV spectra. Therefore, reducing matrix interference could be performed in future studies by optimising sample preparation with different extraction conditions, such as precipitation, solid phase extraction cartridges, liquid-liquid extraction, or liquid phase microextraction [10,24,28]. However, this approach is not the best strategy if the assay will be implemented to lower skilled workers outside a laboratory setting or in a resource-limited practice. Despite the shortcomings exposed in this study, the use of a mobile spectrophotometer is still promising for programmatic implementation of TDM, but strategies should be performed to improve the assay sensitivity and selectivity.

CONCLUSIONS

The proposed mobile microvolume spectrophotometer assay was not sufficiently selective and thereby unsuitable to quantify tenofovir, lamivudine, and efavirenz directly in saliva. High LLOQ values were observed for tenofovir and efavirenz, while the total absorbance of lamivudine and efavirenz in saliva exceeded the linear range for analysis. Further research using different strategies, matrices, or devices could be investigated.

References

[1] F.J. Palella, K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, S.D. Holmberg, Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection, N. Engl. J. Med. 338 (2011) 853–860. https://doi.org/10.1016/B978-0-323-05405-8.00151-0.

[2] B. Punyawudho, N. Singkham, N. Thammajaruk, T. Dalodom, S.J. Kerr, D.M. Burger, K. Ruxrungtham, Therapeutic drug monitoring of antiretroviral drugs in HIV-infected patients, Expert Rev. Clin. Pharmacol. (2016). https://doi.org/10.1080/17512433.2016.1235972.

[3] World Health Organization, Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, Geneva, 2016. https://doi.org/10.1016/j.jped.2014.04.007.

[4] World Health Organization, Consolidated guidelines on HIV prevention, testing, treatment, service delivery and monitoring: recommendations for a public health approach, 2021.

[5] P. Clevenbergh, S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, H. Trout, J.-F. Bergmann, Improving HIV infection management using antiretroviral plasma drug levels monitoring: a clinicians point of view, Curr. HIV Res. 2 (2005) 309–321. https://doi.org/10.2174/1570162043351129.

[6] C.S. Alexander, J.J. Asselin, L.S.L. Ting, J.S.G. Montaner, R.S. Hogg, B. Yip, M. V. O'Shaughnessy, P.R. Harrigan, Antiretroviral concentrations in untimed plasma samples predict therapy outcome in a population with advanced disease, J. Infect. Dis. 188 (2003) 541–548. https://doi.org/10.1086/376835.

[7] Y. Lailulo, M. Kitenge, S. Jaffer, O. Aluko, P.S. Nyasulu, Factors associated with antiretroviral treatment failure among people living with HIV on antiretroviral therapy in resource-poor settings: a systematic review and metaanalysis, Syst. Rev. 9 (2020) 1–17. https://doi.org/10.1186/s13643-020-01524-1.

[8] D. Cattaneo, S. Baldelli, V. Cozzi, E. Clementi, D.J. Marriott, C. Gervasoni, Impact of therapeutic drug monitoring of antiretroviral drugs in routine clinical management of people living with HIV, Ther. Drug Monit. 42 (2020) 64–74. https://doi.org/10.1097/ftd.0000000000000684.

[9] H.J. Galena, Complications occurring from diagnostic venipuncture, J. Fam. Pract. (1992).

[10] V. Avataneo, A. D'Avolio, J. Cusato, M. Cantù, A. De Nicolò, LC-MS application for therapeutic drug monitoring in alternative matrices, J. Pharm. Biomed. Anal. 166 (2019) 40–51. https://doi.org/10.1016/j.jpba.2018.12.040.

[11] J.-W.C. Alffenaar, E.M. Jongedijk, C.A.J. van Winkel, M. Sariko, S.K. Heysell, S. Mpagama, D.J. Touw, A mobile microvolume UV/visible light spectrophotometer for the measurement of levofloxacin in saliva, J. Antimicrob. Chemother. 76 (2021) 423–429. https://doi.org/10.1093/jac/dkaa420.

[12] H.Y. Kim, E. Ruiter, E.M. Jongedijk, H.K. AK, B.J. Marais, B. PK, S. Sawleshwarkar, D.J. Touw, J.-W. Alffenaar, Saliva-based linezolid monitoring on a mobile UV spectrophotometer, J. Antimicrob. Chemother. 76 (2021) 1786–1792. https://doi.org/10.1093/jac/dkab075.

[13] S. Mohamed, H.C. Mvungi, M. Sariko, P. Rao, P. Mbelele, E.M. Jongedijk, C.A.J. van Winkel, D.J. Touw, S. Stroup, J.W.C. Alffenaar, S. Mpagama, S.K. Heysell, Levofloxacin pharmacokinetics in saliva as measured by a mobile microvolume UV spectrophotometer among people treated for rifampicin-resistant TB in Tanzania, J. Antimicrob. Chemother. 76 (2021) 1547–1552. https://doi.org/10.1093/jac/dkab057.

[14] T.R. Zijp, Z. Izzah, C. Åberg, C.T. Gan, S.J.L. Bakker, D.J. Touw, J.F.M. van Boven, Clinical value of emerging bioanalytical methods for drug measurements: a scoping review of their applicability for medication adherence and therapeutic drug monitoring, Drugs. 81 (2021) 1983–2002. https://doi.org/10.1007/S40265-021-01618-7.

[15] V. de Lastours, J. Fonsart, R. Burlacu, B. Gourmel, J. Molina, Concentrations of tenofovir and emtricitabine in saliva : implications for preexposure prophylaxis of oral HIV acquisition, Antimicrob. Agents Chemother. 55 (2011) 4905–4907. https://doi.org/10.1128/AAC.00120-11.

[16] P. Courlet, L.A. Decosterd, J.A. Brown, S.A. Saldanha, C. Marzolini, M. Cavassini, M. Stoeckle, C. Csajka, N.D. Labhardt, A. Calmy, Emtricitabine and lamivudine concentrations in saliva: a simple suitable test for treatment adherence, J. Antimicrob. Chemother. 74 (2019) 2468–2470. https://doi.org/10.1093/jac/dkz181.

[17] A. Theron, D. Cromarty, M. Rheeders, M. Viljoen, Determination of salivary efavirenz by liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 2886–2890. https://doi.org/10.1016/j.jchromb.2010.08.051.

[18] U.S. Department of Health and Human Services, Food and Drug Administration, Bioanalytical Method Validation Guidance for Industry, 2018.

[19] European Medicines Agency, Guideline on Bioanalytical Method Validation, 2011.

[20] K.D.S. Nanda, K. Ranganathan, K.M. Umadevi, E. Joshua, A rapid and noninvasive method to detect dried saliva stains from human skin using fluorescent spectroscopy, J. Oral Maxillofac. Pathol. 15 (2011) 22–25. https://doi.org/10.4103/0973-029X.80033.

[21] J. Østergaard, UV/Vis Spectrophotometry and UV Imaging, in: A. Müllertz, Y. Perrie, T. Rades (Eds.), Anal. Tech. Pharm. Sci., 2016: pp. 3–27. https://doi.org/10.1007/978-1-4939-4029-5_1.

[22] A. Savitzky, M.J.E. Golay, Smoothing and differentiation of data by simplified least squares procedures, Anal. Chem. 36 (1964) 1627–1639.

[23] P.K. Drain, R.W. Kubiak, O. Siriprakaisil, V. Klinbuayaem, J. Quame-Amaglo, P. Sukrakanchana, S. Tanasri, P. Punyati, W. Sirirungsi, R. Cressey, P. Bacchetti, H. Okochi, J.M. Baeten, M. Gandhi, T.R. Cressey, Urine tenofovir concentrations correlate with plasma and relates to TDF adherence: a randomized directly-observed pharmacokinetic trial (TARGET study), Clin. Infect. Dis. (2019). https://doi.org/10.1093/cid/ciz645.

[24] L.E. Hermans, M. Nijhuis, H.A. Tempelman, T. Houts, R. Schuurman, D.M. Burger, A.M.J. Wensing, R. Ter Heine, Point-of-care detection of nonadherence to antiretroviral treatment for HIV-1 in resource-limited settings using drug level testing for efavirenz, lopinavir, and dolutegravir: a validation and pharmacokinetic simulation study, J. Acquir. Immune Defic. Syndr. 87 (2021) 1072–1078. https://doi.org/10.1097/QAI.0000000000002681.

[25] H.C. Koenig, K. Mounzer, G.W. Daughtridge, C.E. Sloan, L. Lalley-Chareczko, G.S. Moorthy, S.C. Conyngham, A.F. Zuppa, L.J. Montaner, P. Tebas, Urine assay for tenofovir to monitor adherence in real time to tenofovir disoproxil fumarate/emtricitabine as pre-exposure prophylaxis, HIV Med. 18 (2017) 412–418. https://doi.org/10.1111/hiv.12518.

[26] M. Gandhi, P. Bacchetti, W.C. Rodrigues, M. Spinelli, C.A. Koss, P.K. Drain, J.M. Baeten, N.R. Mugo, K. Ngure, L.Z. Benet, H. Okochi, G. Wang, M. Vincent, Development and validation of an immunoassay for tenofovir in urine as a real-time metric of antiretroviral adherence., EClinicalMedicine. 2–3 (2018) 22–28. https://doi.org/10.1016/j.eclinm.2018.08.004.

[27] F. Rahimi, S. Chatzimichail, A. Saifuddin, A.J. Surman, S.D. Taylor-Robinson, A. Salehi-Reyhani, A review of portable high-performance liquid chromatography: the future of the field?, Chromatographia. 83 (2020) 1165–1195. https://doi.org/10.1007/s10337-020-03944-6.

[28] M. Dehghani Mohammad Abadi, N. Ashraf, M. Chamsaz, F. Shemirani, An overview of liquid phase microextraction approaches combined with UV-Vis spectrophotometry, Talanta. 99 (2012) 1–12. https://doi.org/10.1016/j.talanta.2012.05.027.

Declarations

Acknowledgements

The authors would like to thank all volunteers for the saliva samples, Department of Analytical Chemistry, University of Groningen, the Netherlands for preparing Molybdovanadate reagent, and the Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT, The Hague, the Netherlands) for supplying efavirenz.

Author contributions

Zamrotul Izzah: Conceptualization, Methodology, Formal Analysis, Visualization, Writing – original draft.
Frank Pierik: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – review and editing.
Leanne Ambrosio: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – review and editing.

Erwin M. Jongedijk: Methodology, Formal Analysis, Validation, Writing – review and editing.
Christoffer Åberg: Conceptualization, Supervision, Writing – review and editing.
Daan J. Touw: Conceptualization, Supervision, Writing – review and editing.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Zamrotul Izzah is supported by the LPDP scholarship (The Indonesian Endowment Fund for Education, Ministry of Finance of Republic of Indonesia) during her PhD trajectory at the University of Groningen, The Netherlands. The supporting source was not involved in the study design, data collection, analysis or writing of the paper.

Data availability
The data that supports the findings of this study are available within the manuscript and its supporting information files.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement
An institutional review board letter was issued by the medical ethics review board of the University Medical Center Groningen (Ref. M23.318050/2023 dated 21 July 2023). They declared that the study fulfilled all the requirements for patient anonymity and was in agreement with regulations applied in the study site for publication of patient data. All healthy individuals gave their written consent prior to saliva collection.