Determination of Lamivudine, Zidovudine, and Nevirapine in Capillary Blood Sampled on Filter Paper by LC

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Abstract

A bioanalytical method for determination of lamivudine (3TC), zidovudine (AZT), and nevirapine (NVP) in 100 µL capillary blood applied onto sampling paper has been developed and validated. The antiretroviral drugs (ARV) were analyzed by reversed phase gradient liquid chromatography with UV detection. Separation was performed on a Zorbax SB C_8 (250 × 4.6 mm) column with a two-step gradient: (i) methanol–0.05 mol/L acetic acid-sodium acetate buffer (pH 3.95, 15:85 v/v) and (ii) methanol–0.05 mol/L acetic acid-sodium acetate buffer (pH 3.95, 50:50 v/v) with a flow rate of 1.0 mL/min. UV detection was performed at 260 nm. Total assay precisions were 6.3, 4.7, and 4.9% for 3TC at 0.34, 0.69, and 3.9 µg/mL, and 5.1, 5.5, and 3.2% for AZT at 0.40, 0.80, and 4.5 µg/mL. For NVP, total assay precisions were 5.2, 8.3, and 3.5% at 2.6, 4.5, and 8.8 µg/mL. Lower limit of quantifications (LLOQ) were 0.11 and 0.13 µg/mL for 3TC and AZT where the precisions were 2.0% for both the analytes. For NVP, LLOQ was 1.3 µg/mL where precision was 2.6%. Concentrations were determined for 10 h for two subjects receiving standard twice daily antiretroviral therapy containing 3TC, AZT, and NVP. Maximum 3TC concentrations were 2.5 and 2.8 µg/mL for subject 1 and 2, respectively. For AZT, maximum concentrations were 1.8 and 1.1 µg/mL while being 15 and 9.6 µg/mL for NVP. Pre-dose trough concentration of NVP was 11 µg/mL for subject 1 and 9.6 µg/mL for subject 2.

Introduction

In 2006, 39.5 million people were living with human immunodeficiency virus (HIV) with more than 60% being in sub-Saharan Africa (1). The World Health Organization (WHO) recommends first-line antiretroviral therapy (ART) to be constructed of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse trancriptase inhibitor (NNRTI). One such regimen is zidovudine (AZT), lamivudine (3TC), and nevirapine (NVP), which are all available as three-drug fixed-dose combination (2).

The knowledge of drug concentrations can be of benefit for patients, especially pregnant women and children (3). If inadequate drug concentration is determined, it may reflect an insufficient dose for a patient or can be due to non-compliance among other reasons. Determining the antiretroviral drug concentration can also provide some means to prevent the development of viral resistance to drugs (4). Drug interactions that might occur between antiretrovirals and other drugs present may also require monitoring of concentrations (5). Drug determination has mainly been focused on NNRTIs and protease inhibitors (PIs) because current understanding is that the measurement of drug concentration might be of little value for NRTIs. Intracellular activation is required of the NRTIs to the active triphosphate metabolite with plasma concentration of the parent drug correlating poorly with the triphosphate concentration (3). However, concentration determinations of NRTIs can be of clinical value (6), for instance when clinical studies are performed in resource-limited countries (7).

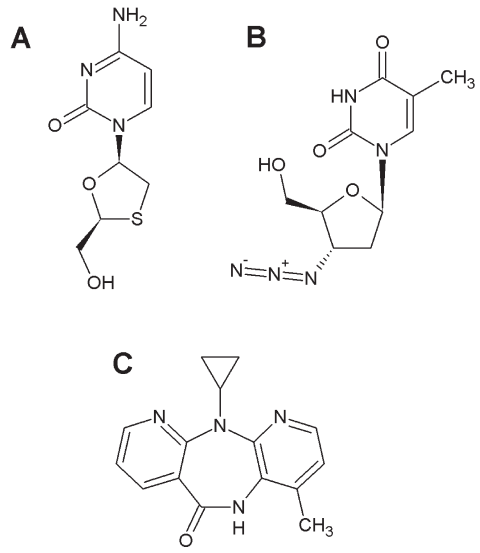

Figure 1. Structures of lamivudine (3TC) (A), zidovudine (AZT) (B), and nevirapine (NVP) (C).

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In low-income countries, samples may be collected at sites remotely located from the laboratory. Storage and transportation might be problematic when using human plasma as well. Sampling of capillary blood has important advantages for patients as only 100 µL capillary blood is needed, and no venipuncture is necessary. When using dried blood spots (DBS), samples can be sent by ordinary mail to the laboratory performing analysis. The risk of HIV virus transmission is almost eliminated as active virus concentration (infectivity titre) is greatly reduced when the blood is dried (8–10). Hepatitis virus may survive for an extended period of time in dried blood (8). However, transmission of HIV or hepatitis virus when handling DBS is highly unlikely (9). Prior to analysis, precautions must be taken (i.e., heat-deactivation), thereby completely inactivating the HIV viruses and significantly reducing hepatitis viral activity (10,11). A fairly simple method without the use of mass spectrometry for sampling followed by determination of antiretrovirals in DBS is highly desirable.

There are several bioanalytical methods for the determination of antiretrovirals in biological fluids, including NRTIs, mostly for measurements in human plasma (Table I). Liquid chromatographic (LC) tandem mass spectrometric (MS–MS) methods have been described for simultaneous determination of several antiretroviral drugs (12,13). LC methods with UV detection have also been described (14–17). Two methods have been published for determination of antiretroviral drugs in DBS: radioimmunoassay has been used to measure AZT only (18), and an LC–MS–MS method has been described for simultaneously determining several PIs and NNRTIs but none of the NRTI drugs (19). Therefore, a method for simultaneously determining 3TC, AZT, and NVP in DBS by gradient LC–UV has been developed. To the best of our knowledge, there are no previously published methods determining these three antiretroviral drugs simultaneously in DBS. The method described in this paper has been validated with respect to accuracy, precision, concentration-response relationship, selectivity, stability, and extraction recovery, according to the Food and Drug Administration (20). The method has been evaluated by determining 3TC, AZT, and NVP concentrations in two patients.

Table I. Comparison of Methods for the Determination of 3TC, AZT, and NVP*

Ref.*	Analytes	LLOQ, LOQ, or LOD (µg/mL)	Matrix	Sample volume (µL)	Method**	Run time (min)
12	3TC/NVP	0.025/0.050†	plasma	500	I: RP C_{18} LC–MS–MS	4.5
13	3TC/AZT	0.0005‡	plasma	500	G: RP C_{18} LC–MS–MS	18
14	3TC/AZT/NVP	0.005/0.025/0.005‡	plasma	600	G: RP C_{18} LC–UV	40
15	3TC/AZT	0.015†	plasma	500	G: RP C_{18} LC–UV	24
16	AZT/NVP	0.025/0.005‡	plasma	250	I: RP C_{18} LC–UV	10
17	3TC/AZT/NVP	0.059/0.058/0.053‡	plasma	1000	I: ion-pair RP C_8 LC–UV	16
18	AZT	0.024§	DBS	110	radioimmunoassay	–
19	NVP	0.102‡	plasma/DBS	100/5	G: RP C_{12} LC–MS–MS	8

* Additional antiretrovirals are determined in references 12, 13, 14, 15, and 19.
† Values correspond to LLOQ. ‡ Values correspond to LOQ.
§ Values correspond to LOD. ** I = isocratic; G = gradient

Table II. ASPEC SPE Procedure for 3TC, AZT, and NVP in 100 µL DBS

SPE-step	Liquid dispensed	Dispensing volume (µL)	Dispensing flow rate (µL/min)	Pressuring air volume (µL)
Conditioning	DIW*	2000	1000	500
Sample loading	sample	5000	500	2000
Washing	NH_4OH–DIW (2:98, v/v)	1000	1000	8000
Elution	Methanol–DIW (70:30, v/v)	1000	500	3000

* DIW = deionized water

Experimental

Chemicals and materials

3TC and AZT were provided by Glaxo Smith Kline (Hertfordshire, UK), and NVP was from Boehringer Ingelheim (Ridgefield, CT). Purity of standards were > 98%. Molecular structures are shown in Figure 1. Deionized water was prepared by a Milli-Q deionized water system (Millipore, Bedford). Drug-free venous heparinized blood was obtained from the Department of Blood Transfusion at Falun Central Hospital (Falun, Sweden). Sampling paper used for the preparation of DBS was Whatman 3MM Chr (Whatman International, Maidstone, UK).

Instrumentation and Chromatographic conditions

Solid-phase extraction (SPE) was performed using polymer (Oasis HLB) columns (30 mg, 1 mL, Waters, Milford, MA). This sorbent contains styrene-divinylbenzene with *N*-vinylpyrrolidone groups added. An automated SPE system, ASPEC XL 4 with Gilson 735 Sampler software v4.04 (Gilson, Middleton, WI) was utilized. The ASPEC uses a positive air pressure in order to push fluids through the column. The LC system consisted of a Waters 600E Multisolvent delivery system (Waters, Milford, MA), a Gilson 234 autoinjector (Gilson, Middleton, WI), and a Spectra 100 UV detector (Spectra Physics, Newport Corporation, Irvine, CA) at 260 nm. Data acquisition was performed using Data Apex Clarity Chromatography station for windows v2.3.0.188 (DataApex Ltd, Prague, Czech Republic). Analytes were separated with a two-step gradient: A, methanol–0.05 mol/L acetic acid-sodium acetate buffer (pH 3.95, 15:85 v/v); and B, methanol–buffer (pH 3.95, 50:50 v/v) at 1.0 mL/min on a Zorbax SB C_8 (250 × 4.6 mm, 5 µm) column. Mobile phase was 100% A for the first 6 min, followed by 100% B, and back to initial conditions of 100% A at 15 min. Buffer was prepared by dissolving 1.0 g anhydrous sodium acetate and 3.75 mL acetic acid 100% p.a. in 1 L deionized water. Statgraphics Plus for Windows 4.0 (Herndon, Virginia) was used for evaluation of regression models. Calculation of distribution coefficients (LogD) has been performed with ACD/ChemSketch with pK_a and LogD batch 9.04 from Advanced Chemistry Development Inc. (Toronto, Ontario, Canada).

Preparation of calibration standards and QC samples

Stock solutions of 3TC (287 µg/mL) and AZT (334 µg/mL)

were prepared in Milli-Q deionized water. NVP stock solution (1.33 mg/L) was prepared in methanol. Stock solutions were used to prepare working solutions containing all analytes (ARV working solutions) in Milli-Q deionized water. All solutions were stored dark at 4°C. ARV working solutions were added to blank venous heparinized blood to obtain calibration standards of 3TC (0.11–4.3 µg/mL), AZT (0.13–5.3 µg/mL), and NVP (1.3–9.3 µg/mL); all together eight different concentrations were obtained. Low, medium, and high quality control samples (LQC, MQC, and HQC) used for the determination of accuracy, precision, and recovery were prepared in the same manner (3TC 0.34, 0.69, and 3.9 µg/mL; AZT 0.40, 0.80, and 4.5 µg/mL; NVP 2.6, 4.5, and 8.8 µg/mL). All spiked samples contained 4% working solution (v/v) with the ARV working solution added being 25 times more concentrated as compared to the calibrator or the quality control (QC) sample being prepared. 100 µL spiked blood was applied on sampling paper, left to dry at ambient temperature over night, and stored at –86°C in plastic bags until analysis. Blank DBS samples were similarly prepared.

Sample preparation

DBS were cut out in whole and placed in polypropylene tubes (DBS slightly folded). 5000 µL extraction fluid consisting of methanol–4 mM zinc sulphate 1:99% (v/v) was added to each tube, and samples were shaken on a mechanical shaker for 15 min. The liquid phase was decanted into new polypropylene tubes and loaded onto SPE columns by the ASPEC. Extraction procedure for the ASPEC XL is shown in Table II. Elutes were collected in borosilicate glass tubes, evaporated at 70ºC under a stream of air, and reconstituted in 200 µL methanol–Milli-Q deionized water (5:95 v/v). 100 µL was injected into the LC system.

Table III. Co-Administered Pharmaceuticals Evaluated for Method Selectivity. Retention times are included in parenthesis, NI = not interfering, ND = not detected, R = removed during extraction from DBS and SPE. 3TC elutes at 7.3 min, AZT at 14.0 min and NVP at 19.7 min.

Abacavir (16.0 min*, NI†)	Fluconazole (15.4 min, R‡)	Cycloguanil (16.1 min, NI)
Acetylsalicylic acid (14.1 min, R)	Fluphenazine (ND§)	Pyrazinamide (7.5 min, R)
Amodiaquine (ND)	Furosemide (18.3 min, R)	Pyrimethamine (17.3 min, NI)
Monodesethylamodiaquine (ND)	Isoniazid (6.4 min, R)	Pyronaridine (21.5 min, R)
Amoxicillin (ND)	Loperamide (ND)	Rifampicin (ND)
Atovaquone (ND)	Lopinavir (ND)	Ritonavir (ND)
Caffeine (14.8 min, R)	Lumefantrine (ND)	Streptomycin (ND)
Cetirizine (ND)	Mefloquine (16.9 min, NI)	Stavudine (11.2 min, NI)
Chloroquine (13.6 min, R)	Metronidazole (11.0 min, NI)	Sulfadoxine (14.8 min, R)
Desethylchloroquine (13.4 min, R)	Nifurtimox (17.0 min, NI)	N(4)-acetylsulfadoxine (17.2 min, NI)
Ciprofloxacin (14.1 min, R)	Ofloxacin (13.9 min, R)	Sulfamethoxazole (14.1 min, R)
Dextropropoxyphene (14.0 min, R)	Pantoprazole (20.7 min, R)	N(1)-acetylsulfamethoxazole (16.0 min, NI)
Diazepam (18.3 min, R)	Paracetamol (10.1 min, NI)	Suramin (ND)
Eflornithine (ND)	Piperaquine (ND)	Tafenoquine (ND)
Emtricitabine (10.4 min, NI)	Primaquine (20.7 min, R)	Tenofovir (6.9 min, R)
Ethambutol (ND)	Proguanil (20.5 min, R)	Trimethoprim (13.5 min, R)
Everolimus (ND)	1-(4-chlorophenyl)biguanide (13. 5 min, R)	

* Retention times are included in parenthesis.
† NI = not interfering.
‡ R = removed during extraction from DBS and SPE.
§ ND = not detected.

Method Development

Regression model selection

Optimal regression model for the data was evaluated by applying different regression models and selecting the one with the most suitable accuracy profile. In these profiles, the 90% confidence limits of each mean concentration are included (21). Profiles with limits deviating more than ±15% [at lower limit of quantification, (LLOQ) ±20%] are excluded (20).

Evaluation of regression models was performed in 100 µL DBS by examining calibration curves with concentrations ranging between 0.11–4.3 µg/mL for 3TC, 0.13–5.3 µg/mL for AZT, and 1.3–9.3 µg/mL for NVP with signal-to-noise ratio (S/N) > 5 at the lowest concentration for each analyte. Calibration curves were examined in duplicate over three days. Peak heights at each concentration level were evaluated for normality by inspection of the normal probability plot. An F-test was performed to examine whether variances of peak heights were concentration dependent (i.e, homoscedastic or heteroscedastic). Models evaluated were ordinary linear regression, weighted linear regression ($1/x^{1/2}$, 1/x. $1/x^2$, $1/y^{1/2}$, 1/y, and $1/y^2$), and log-log transformation with linear regression.

Method Validation

Accuracy, precision, LLOQ, limit of detection

Accuracy and precision was determined by analysis of 100 µL DBS prepared from spiked venous blood at the three QC levels in triplicate during five days. Concentrations were determined by calibration curves prepared on the day of analysis, and the intra-, inter-, and total precisions were calculated as well as the accuracy at each concentration level. LLOQ was set as the concentration where analyte response of a spiked sample was five times the response of a blank sample (S/N > 5) and where analyte determination could be made with acceptable precision (20%) and accuracy (±20%) (20). Limit of detection (LOD) was determined at the concentration where S/N > 3.

Calibration curve

Calibration graphs were constructed with analyte peak height plotted as a function of concentration. The regression model determined optimal for the data was applied. Deviations within ±15% of back-calculated concentrations were acceptable (±20% at LLOQ) (20).

Extraction recovery

DBS at three concentrations of analytes (LQC, MQC, HQC) in triplicate were analyzed during five days. Analyte peak height was compared to direct injections at the same concentrations as after

reconstitution. Direct injections were prepared in methanol–Milli-Q deionized water (5:95 v/v) and stored at 4ºC until the day of analysis.

Selectivity

Blank venous DBS samples from nine healthy volunteers were analyzed in order to investigate endogenous compounds that could interfere with the determination of 3TC, AZT, and NVP. Pharmaceuticals used for treatment of opportunistic infections and tropical diseases were analyzed as well as other analytes commonly present (Table III). Direct injections, corresponding to concentrations at two times C_{max}, were injected into the LC system for evaluation of chromatographic interferences. Substances that interfered during chromatography were further evaluated using spiked DBS samples at therapeutic concentrations. These samples were processed as per the sample preparation procedure, including SPE, in order to evaluate whether or not interfering pharmaceuticals were removed in this process.

Stability

3TC, AZT, and NVP stock solution were stored at 23°C (room temperature) for 6 h in order to evaluate bench-top stability. Stock solutions were diluted to 1 µg/mL with methanol–Milli-Q deionized water (5:95 v/v) prior to analysis. If individual peak heights at t = 6 h was within mean ± 15% of t = 0 h peak heights, stock solution was determined to be stable (20).

Stability of 3TC, AZT, and NVP in the analytical procedure was evaluated. Two concentrations of 3TC, AZT, and NVP (LQC and HQC in triplicate) in 100 µL DBS were analyzed. Samples were prepared and kept at room temperature for 0 and 24 h for each step (i.e., in extraction fluid, on SPE column, in elution fluid, evaporated in glass tubes, and in reconstitution fluid). Short-term stability of 3TC, AZT, and NVP in DBS was evaluated at two concentration levels (LQC and HQC) at room temperature for 24 h. Freeze-and-thaw stability of analytes in DBS was determined in the same manner: at low and high QC level. Samples were stored at –86°C and thawed at room temperature for three freeze-and-thaw cycles. Long-term stability of 3TC, AZT, and NVP in DBS was evaluated at two concentration levels (LQC and HQC). Samples were stored at –17, 4, 23 (i.e., room temperature), and 37°C (i.e., tropical temperature). Calibration standards were stored at –86°C. All samples were analyzed in triplicate on day 1, 5, 15, 33, and 60. Stability during viral deactivation/reduction (i.e., heat treatment) was evaluated. DBS samples at three concentration levels, in triplicate, of 3TC, AZT and NVP (LQC, MQC, and HQC) were stored at 50°C for 3 h (11). Calibration graphs were constructed and concentrations determined. If concentrations were within mean ± 15%, stability was verified (Table IV) (20).

Biological application

Two HIV-positive adults on regular antiretroviral treatment (3TC 150 mg, AZT 300 mg, and NVP 200 mg twice daily) with good adherence records were selected. 100 µL capillary blood was sampled and applied on Whatman 3MM Chr filter paper in duplicate at 0, 0.5, 2, 5, 7, and 10 h. DBS samples were transported at room temperature to the laboratory for analysis. Upon arrival, samples were refrigerated and analyzed two and a half weeks after sampling. Before analysis, samples were submitted to viral heat deactivation (i.e., 50°C for 3 h) (11). Validation of 3TC, AZT, and NVP stability was performed prior to analysis (see Stability). This study was approved by the Uganda National Council of Science and Technology (UNCST). Both subjects gave informed consent to participate.

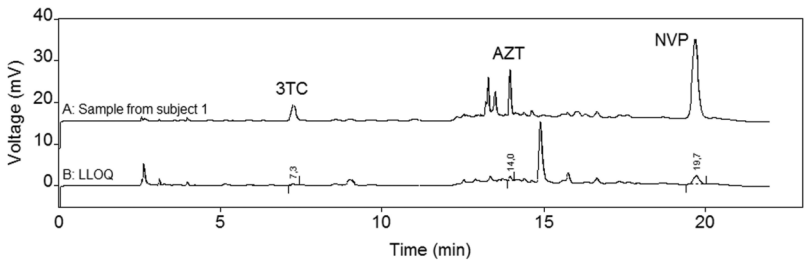

Figure 2. Chromatogram of lamivudine (3TC), zidovudine (AZT), and nevirapine (NVP) in a patient sample 30 min after administration of regular antiretroviral treatment (3TC 150 mg, AZT 300 mg, and NVP 200 mg twice daily) (A) and 3TC, AZT, and NVP at LLOQ (B).

Table IV. Mean Conc. Found, Accuracy, Intra-, Inter-, and Total Assay Precision, and Extraction Recovery for the Determination of 3TC, AZT, and NVP in 100 µL DBS

	Added (µg/mL)	Found (µg/mL)	Intra-assay (%) (n = 15)	Inter-assay (%) (n = 5)	Total assay (%) (n = 15)	Accuracy (%)	Recovery (%) (mean ± SD)
3TC	0.34	0.34	6.0	2.1	6.3	2.2	69 ± 4
	0.69	0.71	4.1	2.3	4.7	3.5	71 ± 3
	3.9	4.1	4.7	1.4	4.9	4.6	72 ± 5
AZT	0.40	0.40	4.5	2.3	5.1	2.8	70 ± 3
	0.80	0.83	4.6	3.0	5.5	4.0	72 ± 3
	4.5	4.8	3.0	1.1	3.2	4.2	75 ± 2
NVP	2.6	2.5	4.9	1.8	5.2	–6.4	58 ± 3
	4.5	4.3	5.9	5.8	8.3	–4.3	55 ± 3
	8.8	9.6	3.3	1.3	3.5	8.0	61 ± 2

Results

Method development

Regression model selection

Ordinary linear regression alone limited the lowest calibration standard to 0.46 µg/mL, 0.47 µg/mL, and 2.0 µg/mL for 3TC, AZT, and NVP, respectively. At lower concentration levels, the confidence limits deviated more than ±20%. With double logarithmic transformation and linear regression, the lowest calibration standard was 0.11 µg/mL for 3TC, 0.13 µg/mL for AZT, and 1.3 µg/mL for NVP. The latter was selected as calibration model based on the extended calibration range as well as a satisfactory accuracy profile (20, 21). This regression model was optimal for all three ARV drugs.

Method Validation

Accuracy, precision, LLOW, LOD

Precision and accuracy data for 3TC, AZT, and NVP is shown in Table IV. LLOQ was 0.11 and 0.13 µg/mL in DBS for 3TC and AZT while precision was 2.0% for both the analytes with deviation of –0.4% and –1.3% (n = 5), respectively. For NVP, LLOQ was 1.3 µg/mL while precision was 2.6%, and accuracy was –1.4%. LOD was 0.069, 0.080, and 0.67 µg/mL for 3TC, AZT, and NVP, respectively.

Calibration curve

Linear calibration graphs were obtained in 100 µL DBS with coefficients of correlation (r > 0.998) (3TC 0.9996 ± 0.0003, AZT 0.9996 ± 0.0003, and NVP 0.9978 ± 0.001, mean ± SD, n = 5). Slopes were 1.012 ± 0.009, 1.021 ± 0.022, and 0.999 ± 0.018 for 3TC, AZT, and NVP, respectively, and intercepts were –0.289 ± 0.017, 0.180 ± 0.024, and –0.458 ± 0.024 (mean ± SD, n = 5). Back-calculated concentrations of calibration standards had deviations of no more than ±11%.

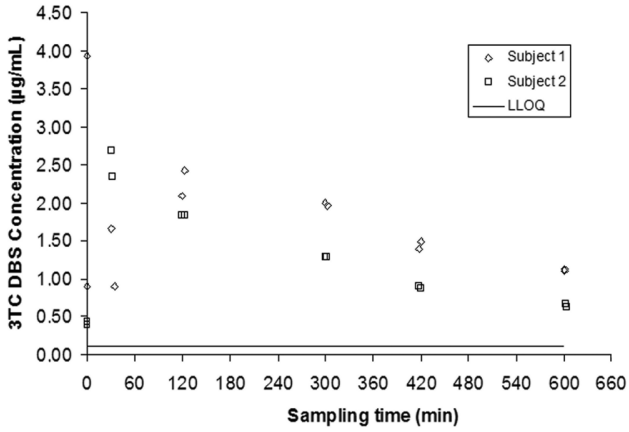

Figure 3. DBS lamivudine (3TC) concentrations of two HIV-infected subjects (n = 2) at different time points after administration of regular antiretroviral treatment (3TC 150 mg, AZT 300 mg, and NVP 200 mg twice daily).

Extraction recovery

Absolute extraction recoveries of 3TC, AZT, and NVP in DBS are found in Table IV. Differences in hydrophobicity between analytes are fairly large with distribution coefficients (LogD) being negative for both 3TC and AZT and positive for NVP. When extracting the analytes from DBS, NVP would benefit from a higher methanol content than utilized. However, if increasing the methanol part, 3TC would be poorly extracted on the SPE column.

Selectivity

Peaks from endogenous compounds with peak heights at least five times lower than at LLOQ were observed in DBS from nine healthy volunteers. These peaks did not interfere with the determination of 3TC, AZT, and NVP at concentrations in the calibration range for each analyte. Due to the presence of these interferences, the LLOQ and LOD were higher than otherwise needed. For 3TC, peak heights in blank samples ranged between 0.011–0.061 mV (n = 9), whereas peak heights for AZT were 0.001–0.123 mV, and 0.089–0.246 mV for NVP. LLOQ was chosen as the concentration resulting in peak height five times the highest endogenous peak co-eluting with the analyte in blank samples. There were no interfering peaks from potentially co-administered drugs observed due to the fact that those drugs resulting in chromatographic interference upon direct injection were removed when extracted from DBS and during SPE (Table III).

Stability

3TC, AZT, and NVP stock solutions were found to be stable at room temperature for at least 6 h. 3TC, AZT, and NVP were stable all through the analytical procedure. However, for NVP an increase or > mean + 15% was noticed when the analyte was kept in extraction fluid at room temperature for 24 h and also when reconstitution of evaporated samples was delayed for 24 h, although the increase did not happen at both QC levels. 3TC, AZT, and NVP were stable during short-term stability, freeze-and-thaw stability and during heat treatment. 3TC and AZT were stable for 60 days at all tested temperatures (–17, 4, 23, and 37ºC). For NVP, stability for 60 days was verified at –17, 4, and 23ºC and for at least 25 days at 37ºC. The analytes were stable during heat treatment at 50ºC for 3 h. Results of the stability experiments are shown in Table V.

Table V. Stability Experiments and Results for 3TC*, AZT†, and NVP‡

Stability experiment	Temp.	Matrix	Time	Found LQC and HQC (CV%) (mg/mL) (Heat: MQC included)		
				3TC	AZT	NVP
Sample prep.	+23°C	Extraction	24 h	0.38 (2.9); 4.1 (4.7)	0.44 (3.0); 4.9 (2.9)	2.9 (4.4); 10 (1.8)
Sample prep.	+23°C	On SPE	24 h	0.33 (3.7); 3.9 (6.6)	0.43 (3.3); 5.1 (6.4)	2.7 (3.9); 11 (6.0)
Sample prep.	+23°C	Elution	24 h	0.36 (5.6); 4.2 (1.1)	0.43 (5.4); 5.0 (0.6)	2.7 (7.5); 10 (3.5)
Sample prep.	+23°C	Evaporated	24 h	0.40 (2.7); 4.2 (2.2)	0.42 (2.9); 5.0 (1.8)	2.9 (4.8); 11 (4.4)
Sample prep.	+23°C	Injection	24 h	0.38 (3.8); 4.2 (9.5)	0.43 (3.9)	2.7 (4.3); 9.4 (9.4)
Short-term	+23°C	DBS	24 h	0.37 (4.1); 4.2 (1.1)	0.44 (5.7); 4.9 (0.8)	2.6 (8.0); 10 (3.2)
Freeze-thaw	–86/+23°C	DBS	3 cycles	0.39 (1.8); 4.3 (5.3)	0.45 (2.8); 4.9 (3.8)	2.8 (4.0); 10 (4.5)
Heat	+50°C	DBS	3 h	0.35 (3.6); 0.67 (0.38); 4.1 (1.2)	0.41 (2.2); 0.77 (1.6); 4.8 (0.7)	2.6 (9.2); 4.2 (1.9); 10 (1.0)
Long term	–17°C	DBS	60 d	0.38 (6.8); 4.3 (1.5)	0.43 (0.8); 4.9 (0.8)	2.6 (1.4); 10 (2.6)
Long term	+4°C	DBS	60 d	0.36 (4.1); 4.1 (3.8)	0.41 (3.4); 4.7 (3.8)	2.5 (6.3); 9.7 (5.6)
Long term	+23°C	DBS	60 d	0.36 (3.5); 3.9 (3.3)	0.41 (3.2); 4.6 (5.2)	2.4 (2.0); 9.1 (9.1)
Long term	+37°C	DBS	60 d	0.34 (4.2); 3.6 (8.1)	0.39 (5.5); 4.5 (1.6)	1.7 (13); 7.7 (3.9)

* 3TC limits for stability are 0.30–0.40 µg/mL at LQC, 0.61–0.82 at MQC, and 3.5–4.7 µg/mL at HQC.
† For AZT, limits are 0.35–0.47 µg/mL at LQC, 0.71–0.96 at MQC, and 4.0–5.4 µg/mL at HQC.
‡ NVP limits are 2.1–2.9 µg/mL at LQC, 3.7–5.0 at MQC, and 8.1–11 µg/mL at HQC.

Biological application

In Figure 2, a chromatogram of 3TC, AZT, and NVP in DBS from subject 1 is shown. The chromatographic run time is 22 min with 3TC eluting at 7.3 min, AZT at 14.0 min, and NVP at 19.7 min. The sample is taken 30 min after the administration of regular antiretroviral treatment (3TC 150 mg, AZT 300 mg, and NVP 200 mg twice daily). Also shown is a chromatogram with 3TC, AZT, and NVP at LLOQ. 3TC, AZT, and

NVP steady state concentrations (n = 2) at different time points are shown in Figure 3, 4, and 5, respectively. QC samples in triplicate at LQC and HQC level varied during assays for 3TC with CV% between 1.1–4.1%, 1.8–4.4% for AZT, and 1.1–5.2% for NVP. 3TC maximum concentration (C_{max}) of subject 1 was 2.5 µg/mL and pre-dose trough concentration (C_{trough}) was 0.89 µg/mL. For subject 2, C_{max} was 2.8 µg/mL, pre-dose C_{trough} was 0.44 µg/mL. AZT maximum concentrations were 1.8 and 1.1 µg/mL for subject 1 and 2, respectively. Pre-dose concentrations were below LLOQ. At approximately 2 h, AZT concentration was at LLOQ for both subjects. Maximum NVP concentration of subject 1 was 15 µg/mL with pre-dose trough concentration being 11 µg/mL. For subject 2, concentrations were 9.6 and 6.1 µg/mL.

Discussion

During method development, the aim was to have a simple and economical assay allowing simultaneous determination of 3TC, AZT, and NVP without the use of mass spectrometry. When injecting extracts directly obtained from DBS samples, resulting chromatograms showed extensive interferences from endogenous peaks. Therefore, SPE was needed for sample clean-up prior to analysis. Whatman 31 ET Chr and Whatman 3MM Chr sampling papers were compared, resulting in similar extraction recoveries and remaining endogenous peaks in chromatograms. Also, as the latter paper was less costly, it was chosen for the assay. To enhance method simplicity, DBSs cut out in whole and DBSs cut into four strips were compared. As no difference was noticed the former was selected, resulting in reduction of preparation time and less handling of infectious material. The sample preparation step of the resulting method described in this paper is straight-forward with cut-out DBSs placed in polypropylene tubes, 5000 µL extraction fluid added, fluids decanted and loaded onto SPE, elutes evaporated, reconstituted, and injected into the LC–UV system.

When developing and validating a bioanalytical method, selectivity is very important to evaluate thoroughly, especially when several other drugs are potentially being co-administered in the prevention and treatment of co-morbidity. Extent of selectivity evaluation differs between published methods. In some cases, selectivity for co-administered drugs appears not to have been evaluated (12,13,17,19). In other publications, possible interferences have been more thoroughly studied, although not for antimalarials (14–16). For methods using tandem mass spectrometry, it appears more common not to evaluate selectivity due to potentially co-administered drugs (12,13,19). It has been assumed that MS–MS detection is highly selective and therefore eliminates interferences from endogenous compounds, but it has now been realized that ion suppression can be caused by sample matrix and interferences from metabolites (22). Therefore, it appears wise to include possibly co-administered drugs during selectivity experiments (or ion-suppression) even for mass spectrometric methods when determining antiretroviral drugs. In this paper, selectivity of drugs used against pneumonia, anti-tuberculosis drugs, antimalarials and some of their metabolites, and antitrypanosomal drugs among others commonly used were studied (Table III) as well as potential interferences from endogenous compounds in the biological matrix. Ideally, metabolites of 3TC, AZT, and NVP as well as metabolites from other antiretroviral drugs should also have been evaluated, but these substances were not obtainable. The triphosphate metabolites of 3TC and AZT are determined in human peripheral blood mononuclear cells after dephosphorylation followed by detection of resulting 3TC and AZT (23). Therefore, it is not likely that the triphosphates would interfere with the described method. NVP has five metabolites: 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine (24). LogD values at pH 3.9 (pH of mobile phase in the described method) are 0.42, 2.04, 2.05, 0.2, and 1.1, while being 1.83 for nevirapine. Theoretically, 2-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine would elute prior to NVP. Still, the differences in LogD between the NVP metabolites and AZT are large, and it is not likely that they will interfere with AZT (LogD –0.53). Additionally, only 12-hydroxynevirapine has been determined in concentrations (0.02–2.0 µg/mL) above the LLOQ of NVP (24).

Drug stability during different circumstances also appears to be evaluated to various extents. Some methods presented seem to lack data on stability of antiretroviral drugs in human plasma in different settings (14,16,17). In other publications, stabilities

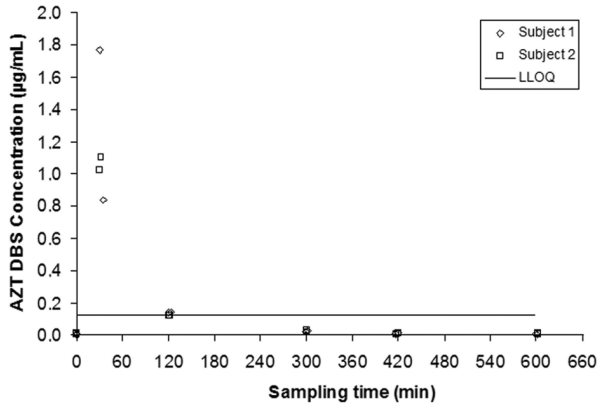

Figure 4. DBS zidovudine (AZT) concentrations of two HIV-infected subjects (n = 2) at different time points after administration of regular antiretroviral treatment.

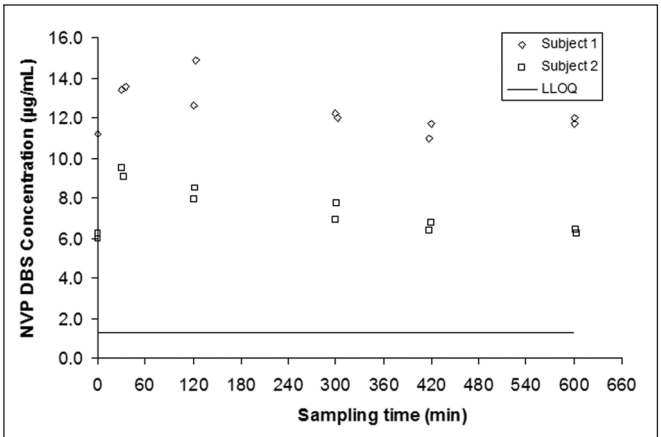

Figure 5. DBS nevirapine (NVP) concentrations of two HIV-infected subjects (n = 2) at different time points after administration of regular antiretroviral treatment.

are evaluated in detail (12,15). NVP, among other antiretrovirals, has been evaluated to some degree in DBS by examining drying times of blood for up to five days, where NVP was determined to be stable (19). However, time from sampling to analysis when performing sampling at remote sites is often longer than five days, and stability is preferably evaluated over much longer period of time. Additionally, evaluation of storage at tropical, refrigerator, and freeze temperatures are of interest. An extensive evaluation of stabilities was performed in this paper as recommended by the FDA guideline (20), aiming to assure correct sample handling prior to analysis. 3TC, AZT, and NVP were stable in most circumstances (i.e., bench top stability of stock solutions, throughout the analytical procedure, during three cycles of freezing and thawing, and during heat treatment). However, differences were noticed when DBS containing 3TC, AZT, and NVP were stored over a couple of months at various temperatures. All three analytes were stable at –17, 4, and 23ºC for 60 days. 3TC and AZT were stable for this period of time at 37ºC as well, but this was not true for NVP where stability was limited to 25 days.

The method described in this paper was applied to determination of drug levels in clinical samples from two subjects on standard twice daily antiretroviral therapy with 3TC 150 mg, AZT 300 mg, and NVP 200 mg. 3TC maximum concentrations (C_{max}) in DBS were 2.5 and 2.8 µg/mL for subject 1 and 2, respectively. These concentrations are higher than found in the literature with C_{max}, in plasma ranging between 0.71–1.8 µg/mL (25,26). C_{max} as well as the time to reach C_{max} (i.e., t_{max}) are affected by food with C_{max} increasing and t_{max} decreasing when fasted (27). 3TC concentrations are also increased with co-administration of co-trimoxazole (28). No information regarding differences or similarities in blood and plasma concentrations has been found in the literature, although this can very well be an explanation for the differences noticed. Target steady state plasma concentration of 0.44 µg/mL for 3TC has been used when evaluating concentration-controlled therapy (29). AZT C_{max} was 1.8 and 1.1 µg/mL for subject 1 and 2, respectively. These values correspond to some values reported for AZT in plasma (16) and are higher than others (25). Similarly to 3TC, AZT concentrations are affected by food intake (27). AZT concentrations may be lower in DBS samples in comparison to concentrations determined in plasma samples (18). For AZT, target steady state plasma concentration of 0.19 µg/mL has been used previously (29). NVP concentrations determined for the two subjects presented in this paper are higher than previously reported in literature. For NVP, C_{trough} was 11 and 6.1 µg/mL for subject 1 and 2, respectively. These concentrations are higher than reported in plasma for a Western population (30). It has been suggested that Ugandan HIV-infected patients have genetic differences resulting in higher NVP concentrations in this population (31). High concentrations of NVP have been noticed and associated with hepatotoxicity, when NVP concentrations are sustained at relatively high levels (> 10 µg/mL) (32). For NVP, the therapeutic target concentration is 3.5 µg/mL in plasma (33).

With the present method, it appears possible to determine 3TC and NVP in DBS samples ranging between the time dose is administered and prior to the next dose. Due to AZT having a short half-life, the time span possible to follow was limited to 2 h, at least for the two subjects studied in this paper. It would be of interest to utilize this method for evaluation of 3TC, AZT, and NVP concentrations in DBS in a study including multiple subjects. It would also be of interest to compare 3TC, AZT, and NVP concentrations determined in DBS with concentrations in plasma. Another focus is determination of NVP concentrations in the Ugandan population. This might require a partial validation as the calibration range may need to be extended.

Conclusion

A bioanalytical method for the simultaneous determination of 3TC, AZT, and NVP in DBS by solid-phase extraction and gradient LC with UV detection has been developed and validated. The method has proven to be accurate, reproducible, and selective. Currently, there are nine co-formulations used in first-line and second-line regimens recommended by the WHO. In six of them, 3TC and/or NVP are included (2). The described method could therefore be useful when evaluating compliance with ARV therapy, especially when sampling is performed in remote areas and/or when sampling paediatric patients.

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