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Simultaneous quantification of zidovudine, stavudine, lamivudine and nevirapine by Micellar Electrokinetic Capillary Chromatography

Quantification simultanée de zidovudine, stavudine, lamivudine et névirapine par chromatographie électrocinétique micellaire

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Abstract – Purpose: Recent improvements to the availability of antiretroviral therapy in Sub-Saharan Africa can be attributed to the generic formulation of antiretroviral drugs. Quality drug surveillance, routine supervision and adherence data are however restricted owing to a fundamental lack of resources in the area. Accordingly we have developed an affordable micellar electrokinetic capillary chromatography (MEKC) method for the simultaneous detection and quantification of zidovudine, stavudine, lamivudine and nevirapine in plasma. **Methods:** The antiretroviral drugs were extracted by solid phase extraction. Various factors influencing separation of the four drugs have been optimized. A buffer consisting of 5 mM sodium tetraborate at pH 9.8, containing 50 mM SDS, 30% methanol and 5% ethanol was found to be particularly suitable and the MEKC method was validated. **Results:** All validation parameters were within the 20% acceptation limit, except for the interday precision of stavudine which required a daily calibration curve. The limit of quantification (LOQ) for zidovudine, stavudine, lamivudine and nevirapine were 0.037, 0.051, 0.029 and 0.028 mg/L respectively and were below the therapeutic concentration ranges of each drug. The optimized MEKC method was successfully applied to 16 human plasma samples. **Conclusion:** Our sensitive and validated method was demonstrated to be suitable for simultaneous detection and quantification of zidovudine, stavudine, lamivudine and nevirapine. This cost-effective method could be of interest for resource limited countries not only for adherence or therapeutic monitoring but also for steady-state pharmacokinetic studies of generic ARV drugs.

Key words: Capillary electrophoresis, drug monitoring, highly active antiretroviral therapy, Micellar Electrokinetic Capillary Chromatography, plasma

Résumé – Objectif : L'accès aux thérapies antirétrovirales en Afrique sub-saharienne s'est récemment nettement amélioré grâce à l'apparition des antirétroviraux sous la forme de génériques. Cependant, la surveillance de la qualité des médicaments, le suivi thérapeutique de routine et les données concernant l'observance restent limités dans ces régions du fait du manque de ressources. Par conséquent, nous avons développé une méthode de chromatographie électrocinétique micellaire (CEM) à faible coût permettant la détection et la quantification simultanée de zidovudine, stavudine, lamivudine et névirapine dans le plasma. **Méthodes :** Une extraction en phase solide a été réalisée afin d'isoler les médicaments antirétroviraux. Différents facteurs influençant la séparation des quatre composants ont été optimisés. Un tampon constitué de tétraborate de sodium 5 mM à pH 9,8, contenant 50 mM de SDS, 30 % méthanol et 5 % éthanol s'avérait le plus adéquat et la méthode CEM a été validée. **Résultats :** Tous les paramètres de validation étaient dans la limite d'acceptabilité de 20 %. La répétabilité calculée pour la stavudine dépassait cependant légèrement cette limite nécessitant l'analyse journalière d'une courbe de calibration. La limite de quantification (LOQ) de la

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zidovudine, de la stavudine, de la lamivudine et de la névirapine étaient respectivement de 0,037, 0,051, 0,029 et 0,028 mg/L, par conséquent, inférieures aux concentrations thérapeutiques de chaque médicament. Seize plasmas humains ont été analysés avec succès par la méthode CEM optimisée. **Conclusion :** Notre méthode sensible et validée a permis la détection et la quantification simultanée de zidovudine, stavudine, lamivudine et névirapine. Cette méthode à faible coût pourrait convenir aux pays en voie de développement non seulement pour le suivi de l'observance du traitement ou le suivi thérapeutique pharmacologique mais aussi pour des études pharmacocinétiques des antirétroviraux génériques.

Mots clés : Électrophorèse capillaire, pharmacovigilance, traitement antirétroviral hautement actif, chromatographie électrocinétique micellaire, plasma sanguin

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1 Introduction

The Human Immunodeficiency Virus (HIV), responsible for the acquired immunodeficiency syndrome pandemic, is estimated to affect 34 million people worldwide, the majority living in Sub-Saharan Africa. Large-scale programs have expanded access to antiretroviral therapy in this region thanks to affordable generic antiretroviral (ARV) drugs. First-line treatment regimens are mainly based on combinations of two nucleoside inhibitors, either stavudine (D4T), lamivudine (3TC) or zidovudine (ZDV), and one non-nucleoside inhibitor like nevirapine (NVP) or efavirenz (EFV). Sub-Saharan African HIV-infected patients are mainly treated with the generic formulation Triomune®, a tablet containing fixed dose combinations of D4T, 3TC and NVP.

Due to a lack of resources and material, ongoing surveillance of generic drug quality is never performed by the authorities before approval in resource-limited countries and routine supervision or adherence data are limited [1, 2]. Non-adherence to antiretroviral drugs or sub-optimal ARV drug plasma concentrations resulting from drug interactions are leading to an accumulation of resistance mutations and subsequent treatment failures [3, 4]. Compliance testing and therapeutic drug monitoring would therefore be beneficial to preserve the limited availability of second line treatment options in these countries [5]. It is notable that some variants of the cytochrome P450 CYP2B6 gene, known to predict decreased plasma NVP or EFV clearance, are more frequent in black African individuals [6–9]. It is therefore crucial to develop affordable ARV quantification methods to allow intensive pharmacokinetic studies in these populations.

Alternate separation techniques such as HPLC, LC-MS/MS, MALDI-TOF/TOF and MALDI triple quadrupole MS/MS have been described [10–15]. However, these costly methods require highly qualified human resources and an extremely well-equipped infrastructure limiting their use in Sub-Saharan Africa.

Capillary electrophoresis (CE) has become an invaluable analytical tool for the determination of drugs in biological matrices and only requires nL volumes of sample amounts [16–19]. CE could indeed serve as the method of choice for separating, analysing and monitoring of drugs in resource-limited countries [20]. Few CE methods have been reported to measure ARV concentrations and were mainly based on micellar electrokinetic capillary chromatography (MEKC) allowing separation of charged and uncharged compounds [21–26]. Sekar and Azhaguvel described two different MEKC methods to separate 3TC and ZDV as well as 3TC, D4T and NVP in pharmaceutical formulations but they encountered difficulties to separate 3TC from the electroosmotic flow (EOF) peak [21,22]. Separation and quantitation of ZDV, NVP and EFV using MEKC was then validated in human serum by two other groups [23, 25]. Tuan *et al.* separated fifteen protease and reverse transcriptase inhibitors in human serum samples with a fast MEKC method [26]. However, the method was not fully validated and showed low recovery for 3TC and only a satisfactory limit of detection (LOD) value for ZDV probably due to a large volume of redissolution (500 μL) of the dried residue to avoid electrophoretic matrix interferences. The aim of our study was to develop a validated MEKC method enabling simultaneous quantification of ZDV, D4T, 3TC and NVP. The method was further applied to plasma samples of 16 HIV-infected patients under antiretroviral therapy.

2 Material and methods

2.1 Reagents and preparation of electrolytes

Zidovudine, lamivudine, stavudine and nevirapine were provided by the National Health Laboratory – Toxicology (Luxembourg). Methaqualone was purchased from Cerilliant Corporation (Texas, USA). Sodium dodecyl sulphate (SDS, $C_{12}H_{25}NaO_4S$), phosphoric acid (H_3PO_4, 85%) and sodium hydroxide (NaOH) were provided by Sigma-Aldrich (Bornem, Belgium). Sodium tetraborate (anhydrous, $Na_2B_4O_7$), boric acid (H_3BO_3), ethanol, methanol (MeOH), acetonitrile (ACN) and ammonia (NH_3, 25%) were obtained from Merck (Darmstadt, Germany). Ammonium chloride (NH_4Cl) was purchased from VWR BDH Prolabo (Leuven, Belgium). High quality ultrapure water was gained from Millipore's AFS 8 Q-Guard A System (Brussels, Belgium).

Ammonia (NH_3, 25%) was added to a saturated solution of ammonium chloride (NH_4Cl) to obtain an ammonium buffer with a pH equal to 9.5. Phosphate buffers at 50 and 100 mM were prepared from H_3PO_4 and adjusted to pH 4.5 and 2.5, respectively. Different MEKC buffers were evaluated: a first buffer containing 12.5 mM sodium tetraborate, 15 mM boric acid, 90 mM SDS and 5% (v/v) ACN with a pH of 10.8 and a second buffer at pH 9.8 containing 10 mM sodium tetraborate, 20 mM SDS, 30% ACN and 5% ethanol. Finally, the most suitable buffer for the separation of the four ARVs consisted of 5 mM sodium tetraborate (anhydrous) adjusted at pH 9.8

containing 50 mM SDS, 30% (v/v) methanol and 5% (v/v) ethanol. Optimal separation was achieved using a daily fresh prepared buffer solution filtered with a sterile 0.22 μm pore size Millex filter to avoid SDS clusters. During each day, buffer solutions were replaced after 12 sample runs.

2.2 Preparation of spiked samples and collection of specimens

Methanolic standard solutions of ARV compounds (1 000 mg/L) and of methaqualone (100 mg/L) were prepared in screw top clear glass vials and stored at −20 °C. Lymphochek Drug Free Serum (Biorad) was spiked with the ARV standard solutions at a concentration of 10 mg/L for NVP (1% v/v) and 4 mg/L for ZDV, 3TC and D4T (0.4% v/v) respectively and stored at −20 °C before analysis.

Anonymized plasma samples from 16 HIV-infected patients under antiretroviral therapy were provided from routine left-over of the "Centre Hospitalier de Luxembourg" (CHL). Nine patients received the ARV drugs Combivir® (300 mg ZDV and 150 mg 3TC, twice daily) and Viramune® (200 mg NVP, twice daily). Three patients were treated with Zerit® (30 mg or 40 mg D4T depending on patient's weight, twice daily), Epivir® (150 mg 3TC, twice daily) and Viramune® (200 mg NVP, twice daily). Two patients had an EFV regimen (600 mg tablet, once a day) in combination with either ZDV, 3TC (300 mg/150 mg, twice a day) or D4T (40 mg, twice daily), 3TC (150 mg, once a day). The protease inhibitor nelfinavir was given to two patients (5 × 250 mg tablets, twice daily) in combination with D4T (40 mg, twice a day) and 3TC (150 mg, twice daily or 300 mg, once a day).

2.3 Extraction procedure

Waters' Oasis® HLB solid phase extraction (SPE) cartridges were used to extract the ARV from specimens and spiked samples. The cartridges were conditioned with 2 mL of methanol, 2 mL of deionised water and 2 mL of ammonium buffer at pH 9.5. 1 mL plasma was mixed to 1 mL ammonium buffer (pH 9.5). A fixed concentration of 2 mg/L of methaqualone was used as internal standard (IS). The sample mixtures were added to the cartridges and drained slowly under slight vacuum. The sorbents were washed with 2 mL of deionised water and dried under vacuum for 15 min. A second wash with 2 mL of hexane/dichloromethane (95:5; v/v) was performed under vacuum. ARV drugs were eluted with 2 mL of methanol. The extracts were evaporated to dryness at 40 °C under nitrogen flux and the residues were resuspended in 100 μL of methanol. To further eliminate interfering matrix compounds, the suspension was centrifuged at 18 000 g for 6 min. 95 μL of the upper layer was evaporated a second time and reconstituted in 10 μL methanol/water (1:1; v/v) for capillary electrophoresis analysis.

2.4 Instrumental parameters and CE conditioning

The drugs' electrophoretic separation was conducted on a Beckman P/ACE^{TM}MDQ Capillary Electrophoresis System equipped with a diode array detector (Beckman Coulter, Fullerton, CA, USA). The 32 Karat software (version 7.0) was used for data acquisition and parameters modification. A 64.6 cm long polyimide coated fused silica capillary with an effective length of 54.3 cm, an outer diameter of 363 ± 10 μm and an inner diameter of 75 ± 3 μm (Composite Metal Services Ltd., Shipley, UK) was chosen for optimal separation. Prior to each sample analysis, the capillary was rinsed at 25 psi with 0.2 M NaOH for 18 s and conditioned with the MEKC buffer for 42 s. A hydrodynamic absorption was done at 0.5 psi for 10 s. Sample stacking was achieved with a short injection of deionised water at 0.1 psi for 1 s. Analytes were separated by applying a 30 kV voltage for 20 min between the positive pole of the anode (left) and the negative pole of the cathode (right). The resulting current varied between 50 and 55 μA. The capillary was heated at 27 °C. Finally, the capillary was rinsed with 0.2 M NaOH at 25 psi for 90 s. All analytes were scanned by a DAD at a wavelength of 230 nm (scan range: 190 nm–320 nm). The scan data rate was 16 Hz. A high sensitivity filter with a peak width of more than 25 points was chosen for optimal data acquisition. Each spiked sample or specimen was analysed in triplicate.

2.5 Method validation procedure

Validation of the method was performed in terms of linearity, intra- and interday precision and accuracy, recovery, LOD and limit of quantification (LOQ).

Ten drug free serum samples spiked with 1.6 mg/L of ZDV, D4T and 3TC and 4 mg/L of NVP were used to determine intraday precision and accuracy. The interday repeatability and accuracy were determined by measuring six equally spiked samples during five days. The baseline noise from a drug free serum sample spiked with 0.4 mg/L ZDV, D4T and 3TC and 1 mg/L NVP was compared with the peak areas of each analytical compound to define the LOD (signal-to-noise ratio (S/N) > 3) and the LOQ (S/N > 10). Recovery of the analytes after SPE was calculated by comparing the peak areas of the ARVs of a blank serum sample spiked with 0.8 mg/L of ZDV, D4T and 3TC and 2 mg/L of NVP before the SPE with those of a plasma sample spiked after the SPE. The acceptance limit for the different validation criteria was fixed at 20%.

3 Results and discussion

3.1 Development of a new MEKC method for ARV separation

A capillary zone electrophoretic method with a 50 mM phosphate buffer at pH 4.5 was firstly evaluated to separate ZDV, D4T, 3TC and NVP of a spiked serum sample. However, only 3TC and NVP could be separated. An increase of the phosphate concentration (from 50 to 100 mM) and changes of the buffer pH could not improve the separation efficiency.

Different MEKC methods were tested to get an optimal separation of the four ARVs. Based on the work of Sekar and Azhaguvel, a buffer containing 12.5 mM sodium tetraborate and 15 mM boric acid adjusted at pH 10.8, 90 mM SDS and 5% ACN was evaluated [21]. With the same electrophoretic

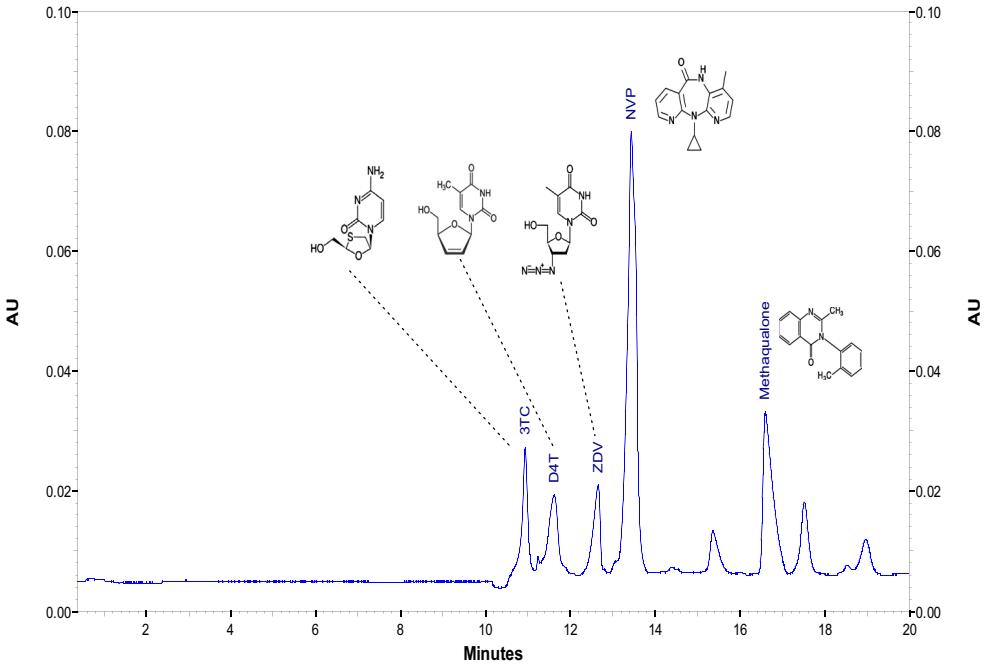

Fig. 1. (Color online) Electropherogram of a spiked serum sample with 1.6 mg/L ZDV, D4T and 3TC and 4 mg/L NVP. The ARVs were scanned at a wavelength of 230 nm.

conditions, the 3TC peak was separated from the EOF peak. Two additional peaks for ZDV and D4T appeared but NVP was undetectable. To modify the EOF and the electrophoretic mobility of the different drugs, additions of 10%, 20% and 30% of ACN were tested to change their solvation [27]. The best result was achieved with 10% ACN but the peaks of ZDV and D4T were no longer separated. Addition of 2% or 5% ethanol and a raise of 5 °C of the capillary temperature (from 25 °C to 30 °C) did not influence the ZDV or D4T electrophoretic migration. Since ZDV and D4T could be separated with a slightly modified version of the buffer used by the group of Pereira, a buffer solution with 10 mM sodium tetraborate (pH 9.8), 50 mM SDS, 30% MeOH and 5% ethanol allowed a good separation between the EOF and the 3TC peak but the capillary temperature had to be raised at 40 °C to get an optimal separation of each ARV drug [25]. To avoid high temperature and the subsequent Joule effect, a decrease of the tetraborate concentration to a final 5 mM was tested and gave similar separation results with a capillary temperature of 27 °C and a voltage of 30 kV. The new MEKC buffer in combination with the method characteristics and the optimal data acquisition parameters were further shown to be effective for the determination of ZDV, D4T, 3TC and NVP in plasma samples.

3.2 Validation of the separation method

An electropherogram of a spiked sample with 1.6 mg/L ZDV, D4T and 3TC and 4 mg/L NVP is shown in figure 1. Preliminary tests showed that better validation results were obtained for ZDV, D4T and 3TC without the IS. For this reason, methaqualone was only used for NVP.

With the peak areas of seven point standards plotted against the concentration, a good linearity of the method was observed. The coefficient of determination was equal to 0.99 for each drug (table I). The calibration curves were linear over the concentration ranges of 0.037–3.2 mg/L, 0.051–3.2 mg/L, 0.029–3.2 mg/L and 0.028–8.0 mg/L for ZDV, D4T, 3TC and NVP, respectively and covered the therapeutic concentration spectrum of the ARVs. Limits of detection and quantification (table I) were found to be lower than in previous studies reported by others, the LODs were 10 to 100 times lower than in the study of Sekar and Azhaguvel [21], Fan and Stewart [23], Pereira *et al.* [25], and Tuan *et al.* [26].

Table I. Coefficient of determination, LOD, LOQ and recovery.

ARV drug	R^{2} [a]	LOD[b] (mg/L)	LOQ[c] (mg/L)	Recovery[d] (%)
Zidovudine	0.997	0.011	0.037	109.1
Stavudine	0.992	0.015	0.051	105.7
Lamivudine	0.989	0.009	0.029	107.7
Nevirapine	0.996	0.008	0.028	87.9

[a] coefficient of determination, [b] S/N > 3, [c] S/N > 10, [d] number of samples $n = 2$.

ARVs extracted from serum with SPE cartridges were successfully recovered (near 100% for ZDV, D4T and 3TC and 88% for NVP, table I). Therefore, to further evaluate the robustness of the MEKC method for NVP, an external quality control (QC) plasma sample containing 3.53 mg/L NVP was added in the validation procedure. The QC was part of the Anti-HIV program launched by the Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT, den Haag, The Netherlands). NVP was successfully quantified: the measured concentration was 3.42 mg/L with an accuracy of 96.9%.

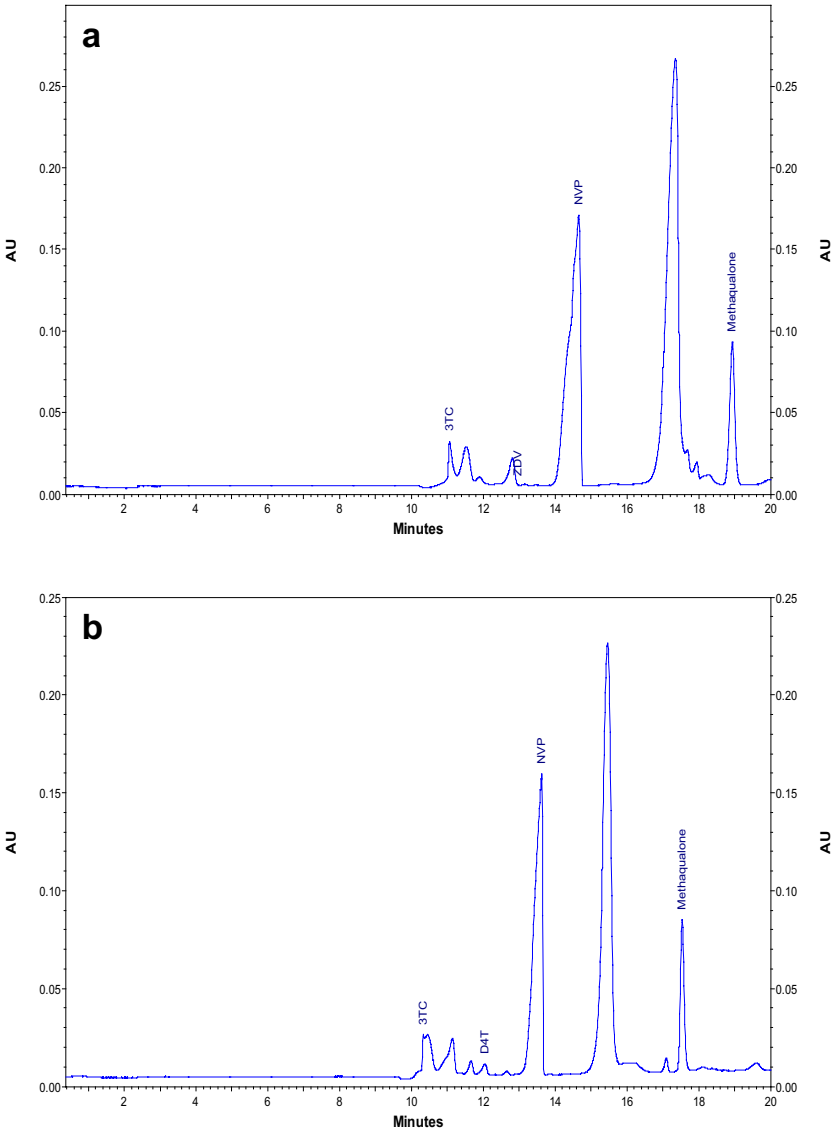

Fig. 2. (Color online) (a) Electropherogram of a plasma sample from an HIV-infected patient treated with zidovudine, lamivudine and nevirapine. The measured drug concentration of ZDV, 3TC and NVP were 0.95 mg/L, 0.97 mg/L and 4.86 mg/L, respectively. (b) Electropherogram of a plasma sample from an HIV-infected patient treated with stavudine, lamivudine and nevirapine. The measured drug concentration of D4T, 3TC and NVP were 0.53 mg/L, 3.03 mg/L and 6.70 mg/L, respectively.

Table II. Intra- and Interday measurements.

ARV drug	Concentration (mg/L)	Intraday (n = 10)		Interday (n = 6)	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Zidovudine	1.6	17.23	99.19	14.07	88.44
Stavudine	1.6	12.63	96.69	22.78	88.11
Lamivudine	1.6	14.88	114.88	10.60	97.03
Nevirapine	4.0	12.15	88.71	15.89	103.39

Intra- and interday repeatability and accuracy were within the acceptance limit, except for the interday precision of D4T (22.78%, table II). Consequently, a calibration curve has to be performed daily before each run.

3.3 Analysis of plasma samples from 16 HIV infected patients under HAART

The validated method was applied to 16 plasma samples from HIV-infected patients under antiretroviral therapy. Figure 2 shows representative electropherograms of a ZDV-3TC-NVP and a D4T-3TC-NVP plasma sample, respectively. The ARV concentrations are presented in table III. ZDV, D4T, 3TC and NVP drug concentrations ranged from 0.02 mg/L to 1.14 mg/L, 0.13 mg/L to 0.86 mg/L, 0.13 mg/L to 3.03 mg/L and 2.34 mg/L to 8.55 mg/L, respectively. One ZDV plasma sample out of 10 had an undetectable drug level. We assumed that the drug concentration was below the detection limit of our method. The sample extract was further analysed with a routine LC/MS-MS technique with a detection limit of 125 ng/L but no ZDV could be detected. These results strongly suggested the absence of the ZDV compound in the patient's

Table III. Quantification of ARVs in plasma samples of treated HIV – infected patients.

Number of patients	HAART[a)]	Daily Dose (mg)	Mean ZDV (SD[b)]) (mg/L)	Mean D4T (SD) (mg/L)	Mean 3TC (SD) (mg/L)	Mean NVP (SD) (mg/L)
1	ZDV-3TC-NVP	600-300-400	0.05 (0.004)	/	0.30 (0.04)	6.50
2	ZDV-3TC-NVP	600-300-400	0.06 (0.01)	/	0.21 (0.02)	8.55 (0.77)
3	ZDV-3TC-NVP	600-300-400	0.06 (0.01)	/	0.13 (0.01)	8.33 (0.67)
4	ZDV-3TC-NVP	600-300-400	1.14 (0.14)	/	0.51 (0.04)	4.70 (0.99)
5	ZDV-3TC-NVP	600-300-400	0.95 (0.48)	/	0.97 (0.27)	4.86 (0.77)
6	ZDV-3TC-NVP	600-300-400	0.40 (0.01)	/	0.16 (0.06)	2.53 (0.06)
7	ZDV-3TC-EFV[c)]	600-300-600	0.08 (0.01)	/	0.45 (0.07)	/
8	ZDV-3TC-NVP	600-300-400	0.03 (0.02; < LOQ)	/	0.25 (0.04)	5.87 (1.73)
9	ZDV-3TC-NVP	600-300-400	0.02 (0.002; < LOQ)	/	0.17 (0.01)	2.34 (1.18)
10	ZDV-3TC-NVP	600-300-400	nd[d)]	/	0.27 (0.01)	3.76 (0.15)
11	D4T-3TC-NVP	80-300-400	/	0.53 (0.02)	3.03 (0.05)	6.70 (0.18)
12	D4T-3TC-NVP	60-300-400	/	0.13 (0.04)	0.15 (0.04)	3.50
13	D4T-3TC-NVP	60-300-400	/	0.86 (0.07)	0.33 (0.03)	3.67 (0.94)
14	D4T-3TC-NFV[e)]	80-300-2,500	/	0.39 (0.01)	0.22 (0.02)	/
15	D4T-3TC-EFV	80-150-600	/	0.35 (0.07)	2.71 (0.16)	/
16	D4T-3TC-NFV	80-300-2,500	/	0.50 (0.08)	1.36 (0.08)	/

[a)] Highly Active Antiretroviral Therapy, [b)] Standard deviation, [c)] Efavirenz, [d)] Not detected, [e)] Nelfinavir.

plasma. The period of time between the last drug intake and the sample collection, pharmacogenetic factors or some drug-drug interactions could explain the absence of ZDV in this sample.

4 Conclusion

The reported MEKC method has been developed to simultaneously quantify ZDV, D4T, 3TC and NVP in plasma samples. The various buffer components and the data acquisition parameters were optimized. The method was validated in terms of linearity, intra- and interday repeatability and accuracy, LOD, LOQ and recovery of each ARV drug after SPE but not fully validated in accordance with the quality assurance schemes ISO 17025 or 15189 or with the 2011 EMEA guidelines [28]. It was further successfully applied to 16 plasma samples from HIV infected patients under ARV treatment. Due to the LOQ values below the therapeutic drug ranges, our method can be proposed for therapeutic drug monitoring of ARVs. Therefore, this cost-effective method could be of interest for resource-limited countries not only for adherence or therapeutic monitoring but also for steady-state pharmacokinetic studies of generic ARV drugs.

Conflicts of interests. The authors have declared no conflict of interest.

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