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Simultaneous determination of antiretroviral drugs in human hair with liquid chromatography-electrospray ionization-tandem mass spectrometry

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Abstract

The determination of the concentrations of antiretroviral drugs in hair is believed to be an important means for the assessment of the long-term adherence to highly active antiretroviral therapy. At present, the combination of tenofovir, lamivudine and nevirapine is widely used in China. However, there was no research reporting simultaneous determination of the three drugs in hair. The present study aimed to develop a sensitive method for simultaneous determination of the three drugs in 2-mg and 10-mg natural hair (Method 1 and Method 2). Hair samples were incubated in methanol at 37 °C for 16 h after being rinsed with methanol twice. The analysis was performed on high performance liquid chromatography tandem mass spectrometry with electronic spray ionization in positive mode and multiple reactions monitoring. Method 1 and Method 2 showed the limits of detection at 160 and 30 pg/mg for tenofovir, at 5 and 6 pg/mg for lamivudine and at 15 and 3 pg/mg for nevirapine. The two methods showed good linearity with the square of correlation coefficient more than 0.99 at the ranges of 416-5000 and 77-5000 pg/mg for tenofovir, 12-5000 and 15-5000 pg/mg for lamivudine and 39-50000 and 6-50000 pg/mg for nevirapine. They gave intra-day and inter-day coefficient of variation less than 15 % and the recoveries ranging from 80.6 to 122.3 % and from 83.1 to 114.4 %. Method 2 showed LOD and LOQ better than Method 1 for tenofovir and nevirapine and matched Method 1 for lamivudine, but there was high consistency between them in the determination of the three drugs in hair. The population

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analysis with Method 2 revealed that the concentrations in hair were decreased with the distance of hair segment away from the scalp for the three antiretroviral drugs.

Keywords

tenofovir; lamivudine; nevirapine; hair; LC-ESI-MS/MS

1. Introduction

Highly active antiretroviral therapy (HAART) consisting of multiple antiretroviral drugs is widely applied to maintain the virological suppression and reduce the morbidity and mortality of patients living with HIV. Strict adherence to HAART tends to favorable virological outcomes [1–3] and poor adherence to HAART seems the most common reason for the failure to achieve the optimal treatment benefits [4]. Therefore, the assessment of the adherence to HAART is important for predicting virological outcomes and avoiding drug resistance.

The adherence to HAART can be evaluated with various methods, such as self-reports, electronic monitoring with the medication event monitoring system, pill counts and pharmacy refill data, the visual analogue scale and the detection of the concentration of antiretroviral drugs in different biomatrices [5]. Among these methods, the detection of drug concentration is the most objective and direct method for the assessment of adherence to HAART [5].

In the previous, drug concentration was mostly performed in biofluids for the assessment of the adherence, such as plasma, peripheral blood mononuclear cell and saliva [6–11]. However, these indices have major limitations in time span and long-term stability when they are utilized to assess long-term adherence to HAART. For instance, drug level in plasma just reflects the adherence in a short-term period (e.g., several hours or days) and is easily affected by external conditions [12, 13]. Alternatively, hair analysis overcomes the limitations in the previous analyses (e.g., blood analysis) [13, 14]. The drug concentration in the 1-cm hair strands closest to the scalp can retrospectively reflect the drug usage over one month if the hair growth rate is 1 cm per month. It also shows high stability in storage and is non-invasive in the sampling [15–17]. Furthermore, it shows high consistency with other indices (e.g., the oral dosage and the drug concentration in plasma) [18, 19]. Therefore, drug concentration in hair may be more superior to the other assessments in predicting long-term adherence [20–22].

High performance liquid chromatography mass spectrometry or tandem mass spectrometry (HPLC-MS or HPLC-MS/MS) were utilized as the primary method for detecting the concentrations of the antiretroviral drugs because of its high sensitivity, specificity and less analysis time [10, 23–25]. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are main ionization methods for HPLC-MS/MS determination. APCI is appropriate for low-polar and non-polar compounds while ESI can be applied to the wider range from low-polar to polar compounds [26]. Therefore ESI is more appropriate for

simultaneous analysis of different antiretroviral drugs with different polarity including strong polarity and low polarity.

Nowadays, lamivudine (3TC) and tenofovir (TFV) or zidovudine in combination with nevirapine (NVP) or efavirenz are adopted as the first-line antiretroviral therapeutic regimen of National Free Antiretroviral Treatment Program, China. Because 3TC, TFV and NVP are the important drugs in the first-line regimen, their simultaneous determination should be conducted. The simultaneous detection had been conducted in blood [27]. However, there was no study reporting simultaneous determination in hair although individual drug concentration was separately determined in several previous studies [17, 22, 28, 29]. Additionally, incubation temperature, incubation duration, hair matrix effect and the position of hair segment in hair shaft are important factors influencing the determination of the concentrations of the three drugs in hair. Previous studies had proven that 37 °C is the optimum incubation temperature for the three drugs [22, 28, 29], and that 14-16 h is the optimum incubation duration for TFV and NVP [28, 29]. However, there was no study reporting the optimization of the incubation duration for 3TC. The effects of hair matrix and the position of hair segment were also unclear.

The present study aimed to develop a sensitive LC-MS/MS method for simultaneous determination of TFV, 3TC and NVP in hair under ESI in the positive mode and multiple reactions monitoring (MRM). In previous studies, different hair weights were used for the detection of the three drugs, such as 2-mg hair for 3TC and NVP [22, 28] and 10-mg or 50-mg hair for TFV [17, 29]. Therefore, 2-mg and 10-mg hair samples were utilized in the present analysis (i.e., Method 1 and Method 2). Then the validated method with good performances was applied to detect the concentrations of the three drugs in natural hair from HIV patients and then investigate the effects of the position of hair segment on the determination of the three drugs in hair.

2. Materials and methods

2.1 Participants and hair collection

Participants were 33 female HIV positive patients (P01-P33) who were randomly recruited from the Guangxi Zhuang Autonomous Region, China. Of these, 31 patients were using 3TC over the past 12 months, 27 patients were taking TFV and 10 patients were using NVP. All participants provided written informed consent prior to inclusion. The present study followed the Declaration of Helsinki and was approved by the Health Science Research Ethics Board of Southeast University. The research was also approved by IRB at University of South Carolina in the USA and Guangxi Center of Disease Control in China.

All participants provided hair strands longer than 12 cm in the posterior vertex region. Prior to use, the first 6-cm hair strands closest to the scalp were cut into six segments with 1 cm in length and the second 6-cm hair strands were cut into three segments with 2 cm in length. The nine hair segments were utilized to probe the change of the concentrations of the three drugs with the distance of hair segment away from the scalp.

2.2 Chemicals and reagents

Standards, lamivudine, tenofovir and nevirapine were purchased from TargetMol (Shanghai, P.R. China). Lamivudine-d3 (3TC- $^{15}\text{N}_2$, ^{13}C) and nevirapine-d3 used as internal standards (IS) for lamivudine and nevirapine were purchased from the Toronto Research Chemicals (Toronto, Canada). Adefovir as IS of tenofovir and methanol (HPLC grade) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium acetate was from Tedia (Fairfield, OH, USA).

Stock solutions of lamivudine, nevirapine and their ISs were prepared in methanol at 1 mg/mL and stored at $-20\text{ }^{\circ}\text{C}$. Tenofovir and adefovir were dissolved in distilled water as stock solution at a concentration of 1 mg/mL and were stored at $4\text{ }^{\circ}\text{C}$. The working solutions of standards were obtained by diluting with methanol to the desired concentrations from 0.1 to 2000 ng/mL. IS working solutions were obtained by diluting with methanol at 20 ng/mL of 3TC-d3, NVP-d3 and 200 ng/mL of adefovir.

2.3 Hair treatment

Hair segments with 1 cm or 2 cm in length were rinsed with 2 mL methanol for 2 min and dried at $50\text{ }^{\circ}\text{C}$ under a blow of pure nitrogen gas. The washing procedure was done twice. After being cut into pieces (1–2 mm) with surgical scissors, hair samples were weighed and transferred to a clean plastic centrifuge tube and then 950 μL methanol and 50 μL IS working solution were added. The mixture was vortex-mixed for 30 s and was incubated for the desired duration (e.g., 16 h) at $37\text{ }^{\circ}\text{C}$ in thermostat water bath. After a 2-min vortex and 5-min centrifugation at 12000 rpm, 800 μL supernatant was transferred into another clean centrifuge tube and evaporated using nitrogen air at $50\text{ }^{\circ}\text{C}$. Finally, the residue was reconstituted with 50 μL mobile phase for next LC-MS/MS analysis.

2.4 Simultaneous analysis of the three drugs

5 μL of the reconstituted solution was injected into a LC-MS/MS system consisting of an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) and 3200 QTRAP tandem mass spectrometer (ABI, Foster City, CA, USA). The Platisil ODS C18 (5 μm , 150 mm $\times$ 4.6 mm; Dikma) reverse phase column was used in the HPLC system for chromatographic separation. A mixture of methanol-water (80:20, v/v) containing ammonium acetate (2 mM) was prepared as mobile phase. It was filtered through micro porous membrane and conducted with over 10 min ultrasonic process after preparation. The column oven temperature was maintained at $40\pm 1\text{ }^{\circ}\text{C}$ and the flow rate was set at 200 $\mu\text{L}/\text{min}$.

Mass spectrometric condition was also optimized for analysis of three drugs. Liquid nitrogen was gasified and used as nebulizing gas. The spectrometer was equipped with electrospray ionization source (ESI) operated in MRM positive mode. Its ion-spray voltage was set at 4500 V. The symmetric heaters were at $400\text{ }^{\circ}\text{C}$. Dwell time was set at 100 ms to monitor all analytes. For gas setting, curtain gas was maintained at 10 psig and collision gas at medium, and both gas 1 (ion source gas) and gas 2 were set at 40 psig. The precursor ion and product ion of individual drug in the optimum condition were shown in Fig. 1. The optimum condition of ionization and fragmentation for three analytes was listed in Table. 1.

2.5 Method validation

The validation was done under the solution of the standards spiked with blank hair matrices that were the hair strands 12 cm away from the scalp from a healthy female adult without the use of the three drugs. Before being incubated in methanol, hair matrices were mixed with 50 μ L working solutions of the standards, 50 μ L working solutions of ISs and 900 μ L methanol, and then processed in the same way as the natural hair samples was done above.

The calibrated curves for both Method 1 and Method 2 were prepared at a final standard concentration with desire ranges. Limit of detection (LOD) was determined at the signal-to-noise ratio of 3. Recovery was determined by comparing concentrations of drugs calculated with calibrated curve relative to the actual concentrations of drug standards. Intra-day and inter-day precisions were validated as the coefficients of variation (CV). Recovery and intra-day and inter-day CV were determined at low, medium and high concentrations that were set at 125, 1250 and 2500 pg/mg for TFV, and at 5, 1250 and 2500 pg/mg for 3TC and 50, 12500 and 50000 pg/mg for NVP in Method 1 and at 100, 1000 and 5000 pg/mg for TFV, and 10, 1000 and 2500 pg/mg for 3TC and 10, 10000 and 50000 pg/mg for NVP in Method 2. Five replicates at the three concentrations were done in a single day for recovery and intra-day CV and repeated on three consecutive days for inter-day CV. Additionally, matrix effect was evaluated as the variation in the sensitivity of the calibration curves with and without blank hair matrix [30].

2.6 Statistical methods

Statistical package SPSS 18.0 for windows was used for statistical analysis. Pearson correlation analysis was done to test the correlation between concentrations (Mean $\pm$ SD) of the drugs detected in 2-mg and 10-mg hair. Repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser correction was conducted for comparison of hair drugs among multiple incubation durations or hair segments. Post hoc multiple comparisons based on least significant difference were conducted for comparison between any two segments.

3. Results

3.1 Chromatography

When being spiked with 2-mg and 10-mg blank hair matrix, the peaks of TFV, 3TC and NVP and their ISs were well resolved as shown in Fig. 2. In the case of 2-mg blank matrix, TFV was firstly eluted at 7.72 min, then 3TC was eluted at 9.38 min and NVP was lastly at 12.41 min. The ISs, adefovir, 3TC-d3 and NVP-d3 were eluted at 7.78, 9.33, and 12.38 min, respectively. In the case of 10-mg blank matrix, the retention time was advanced by 0.1-0.2 min for the three drugs and the ISs. Under the same chromatographic condition, all three drugs in 2-mg and 10-mg natural hair from HIV patients were also separated and showed well-resolved chromatographic peaks with slight longer retention time relative to the standards spiked with blank matrix. The observation of no interfering peaks and lower background noise from the chromatogram showed good sensitivity and selectivity for all three drugs and ISs in the two methods.

3.2 Optimization of hair incubation duration

As shown in Fig. 3, the ratio of the 3TC peak area to the peak area of 3TC-d3 in 10-mg hair was gradually increased till 16 h and tended to be steady from 16 to 30 h where there was no significant difference in the relative ratio of peak area across 16 to 30 h ($F_{(1.496, 2.993)}=0.548$, $p=0.582$, $\eta^2_p=0.215$). Previous studies had proven that 14-16 h was the optimum incubation duration for TFV and NVP in hair [28, 29]. Therefore the present study set 16 h as the optimum duration of hair incubation for simultaneous determination of the three drugs in hair.

3.3 The effect of hair matrix effect on the sensitivity

The hair matrix effect characterized by the suppression or enhancement of analyte's MS response in the presence of blank hair matrix can be evaluated with the variation in the sensitivity (V_s) of standards spiked with and without blank hair matrix, i.e., $V_s=|S-S_m|/S$ where S and S_m are the sensitivity for standards spiked without and with blank hair matrix, respectively [30]. The sensitivity is defined as the instrument response of 1 ng/mL standards solution. It can be calculated with the slope in the fitting linearity of the relative intensity on standard's concentration where the relative intensity was the ratio of the peak area of standard to the IS's peak area.

When standard solutions were spiked with 2-mg blank hair matrix, the sensitivity was slightly increased from 6.1×10^{-3} mL/ng in no blank matrix to 6.7×10^{-3} mL/ng for TFV and decreased from 6.6×10^{-1} to 6.2×10^{-1} mL/ng for 3TC and from 2.2×10^{-1} to 2.1×10^{-1} mL/ng for NVP as shown in Fig. 4. When spiked with 10-mg blank hair matrix, the sensitivity was increased from 1.5×10^{-2} to 1.6×10^{-2} mL/ng for TFV and 6.6×10^{-1} to 8.1×10^{-1} mL/ng for 3TC and slightly decreased from 2.22×10^{-1} to 2.18×10^{-1} mL/ng for NVP. The variation in the sensitivity was 9.8, 4.9 and 6.0 % for TFV, 3TC and NVP in the case of 2-mg blank matrix and was 9.4, 23.7 and 2.2 % in 10-mg blank matrix. The variation in the sensitivity was less than 10 % for the determination of TFV and NVP in both 2-mg and 10-mg hair and of 3TC in 2-mg hair, indicating that there might be no heavy hair matrix effects. However, it was bigger than 10 % in the determination of 3TC in 10-mg hair, indicating there might be heavy hair matrix effect. Additionally, with the increase of the hair weight from 2 to 10 mg, the sensitivity was increased for all three drugs and the variation in the sensitivity had a slight reduction for TFV and NVP, but showed a substantial increase from 4.9 to 23.7 % for 3TC. These results indicated that the increase in the weight of blank hair matrix has no heavy effects on the determination of TFV and NVP, and has heavy effect on the determination of 3TC. However, the increase of the hair weight improved the sensitivity for the determination of 3TC in hair and did not influence the good linearity in the set concentration range as shown in Fig. 4(b).

3.4 Method validation

The validation parameters were separately determined for the two assay methods based on 2-mg and 10-mg hair as listed in Tables 2 and 3. The square of correlation coefficient was more than 0.99 for the two methods, indicating both methods have good linearity in the set concentration range. Limit of detection (LOD) was 160, 4 and 15 pg/mg for TFV, 3TC and NVP in Method 1 and 30, 6 and 3 pg/mg in Method 2. Limit of quantitation (LOQ) was 416,

12 and 39 pg/mg for TFV, 3TC and NVP in Method 1 and 77, 15 and 6 pg/mg in Method 2. Intra-day and inter-day CVs were both less than 15% for two methods. The recovery ranged from 80.6 to 122.3% for Method 1 and from 83.1 to 114.4% for Method 2. These results indicated that both methods have good accuracy and precision, but Method 2 showed LOD and LOQ better than Method 1 for tenofovir and nevirapine in hair and matched Method 1 for lamivudine.

As shown in Fig. 5, there was highly positive correlation in hair 3TC and NVP concentrations between the two methods ($n=31$, $r=0.952$, $p<0.001$ and $n=10$, $r=0.912$, $p<0.001$). Similarly, high correlation was observed for TFV ($n=17$, $r=0.664$, $p<0.01$). The results indicated that there was high consistency between the two methods in the hair determination of the three drugs although there were differences in LOD and LOQ between them. Additionally, Method 2 gave significantly higher 3TC concentration (1.3-fold) than Method 1 (1818 ± 2796 vs 1302 ± 2020 pg/mg, $t_{30}=2.690$, $p=0.012$) due to its higher sensitivity, but there were no differences between them in hair TFV and NVP concentrations ($p>0.05$).

In brief, because Method 2 showed better LOD and LOQ than Method 1, it was applied for detecting the concentrations of the three drugs in natural hair from HIV patients and investigating the effect of the position of hair segment on the concentrations of the three drugs in hair.

3.5 The effect of the position of hair segment

Two HIV patients (P16 and P28) who did not take 3TC over the past 12 months showed hair 3TC concentration below LOD at 6 pg/mg. The similar observation was true for the HIV patients who did not take TFV or NVP over the past 12 months.

Most HIV patients showed a sharp decrease pattern of 3TC concentration in hair segment with the distance away from the scalp except for four HIV patients (i.e., P02, P10, P13 and P24) as shown in Fig. 6(a–b). Their mean 3TC concentrations demonstrated a monotonic decrease from the highest levels in the scalp-nearest segment, then reaching asymptotic-like lower levels in the next five 1-cm segments ($n=26$, $F_{(1.209, 30.228)}=22.507$, $p<0.001$, $\eta^2_p=0.474$) as shown in Fig. 6(c). They dropped by 60.4% from the first segment to the second one, and then dropped by 9.9 % and with average linear decline rate at 58.7 pg/mg per cm ($r=0.990$, $p<0.001$) from the second segment to the sixth one, thereafter there were no significant differences among the other segments ($n=26$, $F_{(1.413, 35.333)}=2.622$, $p=0.103$, $\eta^2_p=0.095$ among the last four segments and $n=26$, $F_{(1.332, 33.288)}=2.633$, $p=0.105$, $\eta^2_p=0.095$ among the last three segments).

As shown in Fig. 7(a–b), TFV concentrations showed significant differences among nine hair segments for 26 patients who used TFV in the past 12 months ($n=26$, $F_{(2.263, 56.580)}=7.338$, $p<0.01$, $\eta^2_p=0.227$) and showed significantly higher levels in the scalp-nearest segment than the other eight segments ($p<0.05$) as revealed by post hoc multiple comparisons. As shown in Fig. 7(c), mean TFV concentration dropped by 68.5 % from the first segment to the second one ($p<0.01$), and then were kept at low levels with no significant differences from the second segment to the ninth one ($n=26$,

$F_{(1.489, 37.233)}=0.978$, $p=0.363$, $\eta^2_p=0.038$ among the segments from the second one to the ninth one; $n=26$, $F_{(2.074, 51.859)}=1.279$, $p=0.288$, $\eta^2_p=0.049$ among the last four segments; $n=26$, $F_{(1.432, 35.802)}=0.069$, $p=0.877$, $\eta^2_p=0.003$ among the last three segments).

As shown in Fig. 8(a), P26 and P28 showed a sharp decrease of NVP concentration from the first hair segment to the other ones, but the other eight patients showed a gradual decrease. As shown in Fig. 8(b), mean NVP concentration dropped by 61.1 % from the first segment to the second one ($p<0.01$) and then maintained at a relatively low level although there was significant difference among the next eight segments ($n=10$, $F_{(1.581, 14.233)}=4.033$, $p<0.05$, $\eta^2_p=0.309$). However, there were no significant differences among the segment from the second one to the sixth one ($n=10$, $F_{(4, 36)}=0.614$, $p=0.656$, $\eta^2_p=0.064$) and no significant differences among the last four segments ($n=10$, $F_{(1.164, 10.476)}=3.041$, $p=0.107$, $\eta^2_p=0.253$) and the last three segments ($n=10$, $F_{(1.083, 9.745)}=2.528$, $p=0.143$, $\eta^2_p=0.219$). The decrease phenomena were called as the washout effect. This was because the decrease in the concentrations of the three drugs in hair might be mainly attributed to hair washing with shampoo solution and water in daily life of human as demonstrated in hair analysis [16].

Additionally, HIV patients showed high inter-individual variations in 3TC concentration with a coefficient of variation at 75.4 % for the first hair segment as shown in Fig. 6(a–b) and a mean coefficient of variation at 82.8 % for the nine segments. Similar inter-individual variations were observed for TFV and NVP concentrations in Fig. 7(a–b) and 8(a) whose coefficients of variation were 103.8 and 99.1 % for the first segment and mean coefficients of variation were 38.6 and 74.3 % for the nine segments.

As shown in Fig. 9(a), HIV patients who took the same oral dosage of 3TC and TFV at 300 mg/day showed significantly higher hair 3TC concentrations than hair TFV concentrations in the first, second and fifth 1-cm segments (i.e., 1897 ± 2993 vs 541 ± 873 pg/mg, $t_{26}=2.195$, $p=0.037$; 474 ± 255 vs 217 ± 385 pg/mg, $t_{26}=3.565$, $p=0.001$; 310 ± 197 vs 142 ± 172 pg/mg, $t_{26}=3.350$, $p=0.002$), but it was not true in the other six segments ($ps>0.145$). They showed significantly lower hair 3TC concentrations than hair NVP concentrations in all the nine segments in Fig. 9(b) and significantly lower hair TFV concentrations than hair NVP concentrations in the first five 1-cm segments in Fig. 9(c), but showed no differences between hair concentrations of TFV and NVP in Segment 6-9 ($ps>0.068$).

4. Discussion

The three antiretroviral drugs, tenofovir, lamivudine and nevirapine in hair were simultaneously determined with LC-ESI-MS/MS in positive mode. To our best knowledge, this successful attempt is the first time in human hair although the three drugs were determined in human plasma based on LC-ESI-MS/MS in negative mode [27].

The present LODs based on 2-mg and 10-mg hair were 160 and 30 pg/mg for TFV. The results matched that (i.e., 60 pg/mg) on 50-mg hair samples in the study of Shah et al. [17], but worse than that (i.e., 10 pg/mg) on 10-mg hair samples in the study of Yang et al. [29]. The present LOQs based on 2-mg and 10-mg hair were 12 and 15 pg/mg for 3TC, which matched that (i.e., 10 pg/mg) on 2-mg hair samples in the previous [22]. Notably, the present

LOQs based on 2-mg and 10-mg hair were 39 and 6 pg/mg for NVP, which was better than that (i.e., 250 pg/mg) on 2-mg hair samples in the literature [28]. These results indicated that the present method enhanced the response of NVP in 2-mg and 10-mg hair and reduced the hair usage to 10-mg hair for the determination of TFV. Therefore the present method would be a good tool for simultaneous quantification of tenofovir, lamivudine and nevirapine in human hair, especially for 10-mg hair.

Based on the previous methods, the present method had done some modifications as follow. Firstly, the present method optimized the incubation duration as 16 h for the extraction of 3TC from 10-mg hair samples (Fig. 3). The optimum duration was also suitable for the extraction of TFV and NVP in hair as demonstrated in previous studies [28, 29]. Secondly, this study utilized methanol as the incubation reagent. In the previous, methanol or other organic reagents and strong acidic or basic aqueous solutions were utilized to incubate hair samples for efficiently extracting antiretroviral drugs [14, 17, 31]. The incubation with methanol was proven to show better extraction recovery than the incubation with strong acidic or basic solutions for TFV [17]. Thirdly, the present method utilized methanol with a high ratio at 80 % as mobile phase for the chromatographic separation of the three drugs in hair. Methanol has the enhancement effect on the ionization of compounds [32]. In the previous, the mobile phase with a lower ratio of organic phase was used, such as 50 % acetonitrile for TFV [17], 45 % methanol for 3TC [22] and 50 % acetonitrile for NVP [28]. Additionally, methanol has higher solubility for nevirapine. The present results showed that the present mobile phase resulted in high signal intensity and the well-resolved sharp chromatographic peaks for the analytes where no interference peaks existed (Fig. 2). Fourthly, this study optimized hair weight for the detection of the three antiretroviral drugs to reduce hair matrix effect. In the previous, 2-mg hair was used for detection of 3TC and NVP and 10-mg and 50-mg hair were used for TFV. The present study used both 2-mg and 10-mg natural hair. The method based on 10-mg hair might suffer from weaker matrix effect than that based on 2-mg hair for TFV and NVP and heavier matrix effect for 3TC (Fig. 4). Despite so, the method based on 10-mg hair showed better sensitivity, LOD and LOQ for all the three drugs as discussed above. In brief, 10-mg hair was selected as the optimum hair weight in the present study.

The present study found that there was large inter-individual variation in the hair concentration of the identical drug among HIV patients who took the same oral dosage every day over 12 month. For example, inter-individual coefficient of variation in the first 1-cm hair segment was 75.4 % for 3TC in Fig. 6(a–b) and 103.8 % for TFV in Fig. 7(a–b) and 99.1 % for NVP in Fig. 8(a). The inter-individual variation might be mainly attributed to the inter-individual variation in the adherence to HAART. Previous study demonstrated a strongly positive correlation between the oral dose of drug and drug concentration in hair [19]. It meant that low drug usage due to low medication adherence or irregular medication might result in the low hair level of drug. Other reasons might be the inter-individual variations in the drug metabolism of HIV patients resulting from their physiological characteristics (i.e., gender and weight) [33, 34] and in the drug incorporation into hair shaft and the drug dissolution out of hair resulting from the irradiation of sunlight and life habits (e.g., hair washing frequency) [16].

The present study also found that there was large inter-drug variation in the hair concentration. For example, patients showed much higher NVP concentrations than hair concentrations of 3TC and TFV in the identical hair segment (Fig. 9). The inter-drug variation might be attributed to their differences in the physiochemical properties, thereby resulting in the inter-drug variations in the drug metabolism and the incorporation mechanism into hair. For example, different drugs show different metabolic rate, which might depend on the metabolic process of different drugs, such as phosphorylation mechanism [35]. Lipophilic molecules can easily penetrate membranes and diffuse into hair [16]. NVP has the highest lipophilicity and the lowest solubility in the water among the three drugs. So NVP is easier to incorporate into hair than the other drugs, resulting in higher concentration in hair.

Finally, the washout effect was observed for all three drugs (Fig. 6–8). The mean drop rate was more than 60% from the first 1-cm segment to the second 1-cm segment for the three drugs. It implied that hair should be cut as close as possible to the scalp if the 1-cm segment closest to the scalp was utilized. Otherwise drug concentration with a big deviation would occur. Additionally, the decrease pattern of drug concentration from the second hair segment to the last one was different for the three drugs. The mean 3TC concentration was dropped with decline rate at 58.7 pg/mg per cm from the second segment to the six one and then stabilized at lower level among the next segments. The mean NVP concentration was maintained at a lower level with a gradual decrease from the second segment to the last one, but the mean TFV concentration was kept at lower stable levels. It implied that the inter-drug variation in the decrease pattern should be considered when hair segment with more than 1 cm was utilized for the assessment of long-term adherence.

In brief, the present findings gave an important implication that inter-individual and inter-drug variations and the washout effect should be taken into accounts when the concentration of antiretroviral drug in hair was utilized as an important tool for the assessment of long-term adherence to HAART.

5. Conclusions

This study had developed the methods based on 2-mg and 10-mg hair (Method 1 and 2) for simultaneous quantification for three antiretroviral drugs, tenofovir, lamivudine and nevirapine. The analysis was performed with HPLC-MS/MS equipped with ESI in MRM positive mode. The validation parameters of the two methods were acceptable according to the FDA guidelines. There was high consistency between the two methods. Method 2 showed better sensitivity, LOD and LOQ than Method 1. Therefore, the method based on 10-mg hair was more suitable for the population analysis in this study. Additionally, this study found that there were high inter-individual and inter-drug variations and the washout effect in the concentrations of the three drugs in hair. There was high inter-drug variation in the decrease pattern of drug concentration in hair segment with the distance away from the scalp. These results would provide valuable guidance for the assessment of long-term adherence to HAART. For example, hair strands should be cut as close as possible to the scalp when the 1-cm segment was utilized to assess the adherence to one-month HAART.

The inter-drug variation in the decrease pattern should be considered when hair segment with more than 1 cm was utilized for the assessment of long-term adherence.

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Highlights

- Simultaneous determination of tenofovir, lamivudine and nevirapine in hair was done.
- Simultaneous detection was performed on LC-ESI-MS/MS in positive mode.
- There were high inter-individual variations in the hair contents of three drugs.
- Hair contents of three drugs showed the washout effect with different patterns.

Figure 1.

Ion pairs of analytes (a-b) tenofovir (TFV), (c-d) adefovir (ADV), (e-f) lamivudine (3TC), (g-h) lamivudine-d3 (3TC-d3), (i-j) nevirapine (NVP) and (k-l) nevirapine-d3 (NVP-d3). (a, c, e, g, i, k) precursor ion and (b, d, f, h, j, l) product ion.

Figure 2.

The chromatograms of analytes in blank hair matrix, blank hair matrix spiked with standard solutions and hair samples from HIV patients. (a-b) TFV, (c-d) 3TC, (e-f) NVP and (g-h) their ISs spiked with blank hair matrix. When spiked with 2-mg blank matrix (figures on the left), the standard concentrations were 500 pg/mg for TFV, 3TC and NVP. The typical concentrations of drugs detected in 2-mg hair samples were 435 pg/mg for TFV, 658 pg/mg for 3TC and 546 pg/mg for NVP. When spiked with 10-mg blank matrix (figures on the right), the standard concentrations were 1000 pg/mg for TFV and 100pg/mg for 3TC and

NVP. The typical concentrations of drugs detected in 10-mg hair samples were 767 pg/mg for TFV, 97 pg/mg for 3TC and 131 pg/mg for NVP. The ISs' concentrations were 200 ng/mL for adefovir and 20 ng/mL for 3TC-d3 and NVP-d3.

Figure 3.
Time courses of the ratio of the 3TC peak area to the peak area of 3TC-d3 in the incubation of 10-mg hair samples from one HIV patient who took 3TC.

Figure 4.

The determined sensitivity for (a-b) tenofovir, (c-d) lamivudine and (e-f) nevirapine spiked with and without 2-mg blank hair matrix (figures on the left) or 10-mg blank hair matrix (figures on the right). S is the sensitivity and R^2 represents the square of correlation coefficient.

Figure 5.

Linear correlations in (a) 3TC and (b) NVP concentrations between the two methods based on 2-mg and 10-mg hair samples from HIV patients.

Figure 6.

The changes of 3TC concentration in 1-cm segment (Segment 1-6) and 2-cm segment (Segment 7-9) with the distance away from the scalp for HIV patients who used 3TC, (a) P01-P15, (b) P17-P27, P29-P32 and (c) mean. P16 and P28 did not take 3TC. P33 was excluded because her hair strands less than 10 mg in weight of Segment 2-9. Mean concentration was obtained from 26 patients because 4 patients (P21, P25, P26 and P30) with hair strands shorter than 12 cm in length and less than 10 mg in weight of Segment 7-9

were excluded. Hair segment 1 is closest to the scalp. Error bar in the figure is standard error mean.

Figure 7.

The changes of TFV concentration in 1-cm segment (Segment 1-6) and 2-cm segment (Segment 7-9) with the distance away from the scalp for patients who used TFV, (a) P01-P10 and P12-P14 and (b) P15-P25, P30 and P31 and (c) mean. TFV mean concentrations were calculated from the 26 patients. Hair segment 1 is closest to the scalp. Error bar in the figure is standard error mean.

Figure 8.

The changes of (a) NVP concentration and (b) NVP mean concentrations in 1-cm segment (Segment 1-6) and 2-cm segment (Segment 7-9) with the distance away from the scalp for 10 HIV patients. Hair segment 1 is closest to the scalp. Error bar in the figure is standard error mean.

Fig. 9.

The comparisons of concentrations in hair between the three drugs in the nine segments. (a) 3TC and TFV among 27 patients for Segment 1-6, among 26 patients for Segment 7-8 and among 23 patients for Segment 9 where HIV patients took 300 mg 3TC and TFV every day. (b) 3TC and NVP among 10 patients for Segment 1-5, among 9 patients for Segment 6, among 8 patients for Segment 7, among 7 patients for Segment 8 and among 6 patients for segment 9 where HIV patients took 300 mg 3TC and 400 mg NVP every day. The 3TC concentration was enlarged by 10 times and the NVP concentration in hair was normalized

to 300 mg every day. (c) NVP and TFV among 8 patients for Segment 1-6 and among 7 patients for Segment 7 and among 6 patients for Segment 8 and among 5 patients for Segment 9 where HIV patients took 300 mg TFV and 400 mg NVP every day. The TFV concentration was enlarged by 10 times and the NVP concentration in hair was normalized to 300 mg every day. Error bar in the figure is standard error mean.

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Table 1

Optimum parameters of ionization and fragmentation and retention time of analytes.

Analyte	Precursor ion/Product ion(Da)	Retention time(min)	DP(V)	EP(V)	CEP(V)	CE(eV)	CXP(V)
TFV*	288.2/176.4	7.34	42.23	2.48	19.48	36.74	4.19
TFV	288.2/136.1	7.38	42.23	2.48	19.48	31.61	5.47
ADV	274.4/162.0	7.25	52.73	5.21	20.07	41.72	3.20
3TC*	230.3/112.1	8.91	27.36	4.94	23.71	28.58	2.42
3TC	230.3/94.6	8.88	27.36	4.94	23.71	26.94	3.36
3TC-d3	233.3/114.7	8.91	40.60	3.56	18.14	16.40	4.18
NVP*	267.1/226.3	12.91	54.01	5.38	21.30	37.39	3.42
NVP	267.1/198.3	12.91	54.01	5.38	21.30	50.75	3.08
NVP-d3	270.1/229.4	12.90	63.43	5.93	24.97	36.61	3.48

Notes: The most sensitive transition (marked as *) was used for quantitation and the other one was used for confirmation. DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy and CXP, collision cell exit potential.

Table 2

Validation parameters of tenofovir, lamivudine and nevirapine for 2-mg hair.

	Tenofovir	Lamivudine	Nevirapine
Calibration curve	$y=0.0003x+0.0265$	$y=0.0249x+0.1008$	$y=0.0084x+0.3633$
R^2 ^a	0.998	0.999	0.999
Linear Range (pg/mg)	416-5000	12-5000	39-50000
LOD (pg/mg) ^b	160	5	15
LOQ (pg/mg)	416	12	39
Intra-day CV (%; n=5) ^{c, d}			
Low	13.9	7.1	8.0
Medium	10.8	10.7	9.0
High	5.6	5.6	8.8
Inter-day CV (%; n=3) ^{c, d}			
Low	11.9	7.4	9.1
Medium	10.0	11.3	8.7
High	5.6	6.5	8.9
Recovery (%; n=5) ^c			
Low	94.7±13.1	109.4±7.8	93.4±7.5
Medium	80.6±8.7	90.5±9.7	92.8±8.3
High	82.0±4.6	122.3±6.8	110.7±9.7

^a R^2 represents the square of correlation coefficient.^bLimits of detection were set at S/N = 3.^cLow, medium and high concentrations were 125, 1250, 2500 pg/mg for tenofovir, 5, 1250, 2500 pg/mg for lamivudine and 50, 12500, 50000 pg/mg for nevirapine, respectively.^dIntra-day and inter-day precision was estimated by coefficient of variation (CV).

Table 3

Validation parameters of tenofovir, lamivudine and nevirapine for 10-mg hair.

	Tenofovir	Lamivudine	Nevirapine
Calibration curve	$y=0.0037x+0.0513$	$y=0.1480x+0.1764$	$y=0.0435x+0.5941$
R^2 ^a	0.995	0.998	0.999
Linear Range (pg/mg)	77-5000	15-5000	6-50000
LOD (pg/mg) ^b	30	6	3
LOQ (pg/mg)	77	15	6
Intra-day CV (%; n=5) ^{c d}			
Low	5.3	13.4	8.2
Medium	11.5	10.0	8.7
High	7.0	7.8	6.5
Inter-day CV (%; n=3) ^{c d}			
Low	8.1	8.9	6.8
Medium	9.9	11.0	7.0
High	8.3	8.6	4.4
Recovery (%; n=5) ^c			
Low	108.1±5.8	100.9±13.5	114.4±13.9
Medium	100.3±11.6	98.6±10.9	103.0±9.0
High	89.2±6.2	83.1±6.5	98.6±6.4

^a R^2 represents the square of correlation coefficient.^bLimits of detection were set at S/N = 3^cLow, medium and high concentrations were 100, 1000, 5000 pg/mg for tenofovir, 10, 1000, 2500 pg/mg for lamivudine and 10, 10000, 50000 pg/mg for nevirapine, respectively.^dIntra-day and inter-day precision was estimated by coefficient of variation (CV).