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Microanalysis of the antiretroviral nevirapine in human hair from HIV-infected patients by liquid chromatography-tandem mass spectrometry

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Abstract

Sufficient drug exposure is crucial for maintaining durable responses to HIV treatments. However, monitoring drug exposure using single blood samples only provides short-term information and is highly subject to intra-individual pharmacokinetic variation. Drugs can accumulate in hair over a long period of time, so hair drug levels can provide drug exposure information over prolonged periods. We now report on a specific, sensitive and reproducible LC-MS/MS method for measuring nevirapine (NVP), a widely used antiretroviral drug, levels in human hair using even a single short strand of hair. Hair samples are cut into small segments and drug is extracted in methanol/trifluoroacetic acid (v/v, 9:1) shaken at 37°C in a water bath overnight, followed by liquid-liquid extraction under alkaline conditions. The extracted samples are then separated on a BDS-C₁₈ column with mobile phase composed as 50% acetonitrile containing 0.15% acetic acid and 4 mM ammonium acetate with an isocratic elution for a total run time of 3 min. and detected by triple quadrupole electrospray multiple reaction mode at precursor/product ion at 267.0>225.9 m/z. Deuterated nevirapine-d5 was used as internal standard. This method was validated from 0.25 to 100 ng/mg using 2 mg hair samples. The accuracies for spiked NVP hair control samples were 98–106% with coefficients of variation (CV) less than 10%. The CV for incurred hair control samples was less than 7%. The extraction efficiency for incurred control hair samples was estimated at more than 95% by repeated extractions. This method has been successfully applied to analyze more than 1000 hair samples from participants in a large ongoing cohort study of HIV-infected participants. We also showed that nevirapine in human hair can easily be detected in a single short strand of hair. This method will allow us to identify drug non-adherence using even a single strand of hair.

Keywords

Antiretroviral drug; Nevirapine; Hair; LC-MS/MS; TDM; Adherence

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Introduction

Adherence to prescribed medications is a key determinate of the success of treatment for many acute and chronic conditions, though nonadherence is a particular problem for long term usage. Nonadherence is also a persistent challenge for the accurate determination of efficacy of interventions in clinical trials research [1, 2]. Combinations of potent antiretroviral therapies (cART) have been successfully used for HIV treatment. Effective cART require high rates of adherence over years of treatment for the durable and effective inhibition of HIV viral replication, but adherence to antiretrovirals (ARVs) can be challenging due to the chronicity of these regimens and not infrequent side effects [3–5]. A suboptimal ARV drug level is a major contributor to virologic failure and the acquisition of viral resistance [3–6]. Viral resistance to antiretroviral drugs imperils both individual treatment outcomes, and poses population risks due to the transmission of resistant HIV [6]. Successful cART can reduce an HIV-infected individual's viral load to an undetectable level and significantly lower the subsequent risk of HIV transmission [7, 8].

Reasons for drug nonadherence are complex and depend on the type of treatment, frequency of dose, specific populations, individual habits, socioeconomic status, concomitant conditions, etc [1]. Current mechanisms to measure adherence each have their limitations. Conventionally used measures of adherence include self-report, pill count, pharmacy records, and microelectronic measurement systems [1]. However, there is no “gold standard” that accurately or reliably measures drug adherence [1]. Adherence as assessed by self-report is usually higher than the actual drug amount taken [2, 9]. So, an objective method for measuring drug exposure is highly desired. Drug exposure not only depends on adherence, but also on other physiopathological factors, such as liver or renal diseases, intestinal surgery, pregnancy etc., that may affect the drug's pharmacokinetics.

Conventionally, drug exposure is measured by the drug concentration in blood (plasma or serum) or urine. However, a single blood or urine sample only provides short term (hours to 1–2 days) information on drug exposure. The difference in trough and maximum concentration (C_{max}) blood levels of some ARV is large, and the drug levels in blood are significantly affected by the time the drug was last taken and time of blood sampling. Some patients may improve adherence before visiting the clinic, i.e. the “white coat” effect, which can lead to atypical estimates of drug exposure [9]. Therefore, a single blood concentration for therapeutic drug monitoring (TDM) of an HIV medication may not provide accurate measures for actual drug exposure during the therapeutic time window. The value of plasma drug level measurements as a means of improving the outcomes of HIV treatment using standard methods has therefore been debated [10–15].

In contrast to the short half life of a drug in plasma or urine, the drug is bound to the matrix of hair as it grows, and remain there over an extended period of time [16–19]. Drug levels measured in hair may provide a unique approach to assessing long-term drug exposure [17–19]. Analysis of drugs by hair segment can also provide historical drug exposure information [16, 17]. Our interest in this approach was first triggered when Bernard and colleagues reported levels of the antiretroviral protease inhibitor, indinavir, in hair were closely correlated with extent of HIV suppression [20, 21]. Duval et al. also found a significant association between HIV-RNA below 50 copies/ml and indinavir concentrations in hair but did not find a similar association with single plasma indinavir levels [22]. The method of measuring indinavir in hair employed by this group used high performance liquid chromatography (HPLC) methods, which lacked sensitivity, so that a large quantity of hair was required for analysis [20], a method that would likely reduce acceptability of routine hair collection in clinical practice. To investigate whether hair levels of ARVs can be used for TDM in HIV patients, we have developed liquid chromatography coupled with tandem

mass spectrometry (LC-MS/MS) methods for analyzing various protease inhibitors, lopinavir, atazanavir, and ritonavir, and a nonnucleoside reverse transcriptase inhibitor (NNRTI), efavirenz, in human hair samples [23,24]. In a large cohort of HIV-infected women, we have shown that lopinavir and atazanavir levels measured in hair are strongly predictive of treatment outcomes over time [24–27].

Nevirapine (NVP) is a NNRTI that is widely used in resource-limited settings and is a mainstay in prevention of mother-to-child transmission of HIV [28–30]. NVP has been reported to cause severe skin rashes or hepatotoxicity, some of which may be related to drug overexposure [31–33]. Therefore, a simple method to monitor NVP exposure could be helpful in the clinical setting. In this paper, we report a microanalysis LC/MS/MS method for measuring NVP levels in human hair using even a single short strand of a hair sample.

Materials and Methods

Materials and Reagents

Nevirapine hemihydrate reference compound and internal standard (IS), deuterated nevirapine-d5, were obtained from USP (Rockville, MD) and the Toronto Research Chemicals (Toronto, Canada), respectively. Acetonitrile, methanol (MeOH), trifluoroacetic acid (TFA) and other solvents or reagents were HPLC grade or analytical grade. Human scalp hair samples were obtained from patients on NVP-based cART in the Women's Interagency HIV Study (WIHS) [33]. Blank human hair samples for negative controls were obtained from healthy volunteers. Hair shampoo and conditioner used for this study were PANTENE Pro-V, Fine Hair Solutions, Flat to Volume Shampoo, USA, and PANTENE Pro-V, Fine Hair Solutions, Flat to Volume conditioner, USA.

Standard solutions

Two independent NVP stock solutions and one IS stock solution were prepared at 1 mg/ml in 50% acetonitrile and stored at -70°C . The high working solution (20 $\mu\text{g/ml}$) of NVP was diluted from the stock solution with 50% acetonitrile. The low working solution (0.5 $\mu\text{g/ml}$) was diluted from the high working with 50% acetonitrile. The working solutions were stored at 4°C . The stock solutions and working solutions were stable at least 6 months. The internal standard working solution containing 0.5 $\mu\text{g/ml}$ of NVP-d5 was prepared by dilution of internal standard stock solution with 50% acetonitrile and stored at 4°C .

NVP extraction from human hair

To test a variety of drug extraction conditions, we collected large amount of scalp hair samples from several HIV patients on NVP-based cART. We first divided hair sample (black color) from one patient into two parts to assess the best method for NVP extraction. One part was chopped into small pieces (about 1–3 mm segments) into a glass container with surgical scissors. The second part of the hair sample was ground to a fine powder by a Frozen Mill™ (6750 model, SPEX CertiPrep, Inc, N.J.) after liquid nitrogen freezing. About 2 mg of hair powder or the finely-cut hair sample was placed into glass test tubes (16×125 mm), then 1 ml of MeOH/TFA (v/v , 9:1) and 50 μl of IS working solution were added and the sample was shaken at 37°C in a water bath overnight ($\sim 14\text{h}$). The MeOH/TFA was evaporated by nitrogen (N_2) gas at room temperature, then 1 ml of 0.2 M sodium phosphate buffer (pH 9.4) was added. The samples were vortexed for 0.5 min., then 3 ml of methyl tert-butyl ether (MTBE)/ethyl acetate (EA) (v/v , 1:1) was added and the samples were vortexed for 1 min., three times, followed by centrifugation at 3000 rpm for 10 minutes. The samples were then frozen in a methanol/dry ice bath, and the organic solvent was poured into another test tube. The organic solvent was then evaporated by N_2 gas. The evaporated

samples were reconstituted with 0.2 ml of 50% CH₃CN, and 10 µl were injected into the LC-MS/MS system for drug analysis.

Liquid chromatography-tandem mass spectrometry

The LC-MS/MS system consists of a Shimadzu LC-10 AD pump, a Waters intelligent Sample Processor 717 Plus autosampler, and a Micromass Quattro LC triple quadrupole tandem mass spectrometer. The mass spectrometer was set to electrospray ionization (ESI) in positive multiple reaction monitoring (MRM) mode. The product ion mass spectra of NVP and IS are shown in Figure 1. The precursor/product transitions (m/z) were at 267.0>225.9 m/z for NVP and 272.0>227.0 m/z for NVP-d5, respectively. The cone voltage and collision energy were 40 V and 25 eV, respectively, for both NVP and IS. The source block and desolvation temperature were 100°C and 400°C, respectively. The HPLC conditions were as follows: The column was a BDS-C₁₈ (100 × 4.6 mm) from Hypersil-Keystone™, and the mobile phase was composed of 50% CH₃CN containing 0.15% acetic acid and 4 mM ammonium acetate. The flow rate was set at 0.8 ml/min and 25% of flow was split into mass system. Data processing was performed using Masslynx 4.0 software.

Method validation

Calibrator, QC and Sample Preparation—The calibration curve and spiked quality control (QC) samples were prepared by spiking the NVP working solutions to 2 mg of cut blank human hair samples in glass tubes (16 × 125 mm). The final drug concentrations for the standard curve were 0, 0.25, 0.5, 1.0, 2.0, 5.0, 10, 25, 50, and 100 ng/mg hair. The low (L), medium (M), and high (H) QC concentrations were 0.75 ng/mg, 5 ng/mg and 70 ng/mg, respectively. The incurred hair QC samples were prepared by cutting the hair of a pooled human hair sample from one of the HIV-positive patients on a NVP based regimen into small segments (1–3 mm) and mixing well in a glass container; this sample was stored at room temperature. During assay validation, approximately 2 mg incurred QC samples were aliquoted to the same type of glass tube, and hair weights were obtained with a Sartorius analytical balance (MC 210P model). The standard curve and QC samples were then processed and analyzed in the same way as the NVP hair sample extraction section described above.

Matrix effect—The matrix ionization suppression or enhancement was evaluated through measuring the Matrix Factor (MF) and IS normalized MF as recommended by a recent FDA/AAPS white paper on drug bioanalysis [35]. Six lots of blank human hair samples were cut into small segments and treated with MeOH/TFA (v/v, 9:1), followed by liq-liq extraction with MTBE/EA by the same procedures as described above. After the evaporation of MTBE/EA, medium QC level NVP (20 µl × 0.5 µg/ml), IS (50 µl × 0.05 µg/ml) and 130 µl of 50% CH₃CN (total volume was 200 µl) were added to the sample tubes. Samples were vortexed for 1 min., then 10 µl were injected into LC/MS/MS system. Control samples were prepared using the same concentration levels of drug and IS, but the hair matrix was absent.

Extraction recovery—The extraction recovery of NVP from hair samples was evaluated in two ways. One was extraction recovery of spiked hair QC samples; the other was repeated extraction of incurred hair samples. The spiked hair sample extraction recovery was carried out as follows: The L, M and H QC concentration levels of NVP were spiked to 2 mg blank cut hair samples, then the samples were dried in the air hood at room temperature. These samples were treated with 1 ml of MeOH/TFA (v/v, 9:1), shaken at 37°C in a water bath overnight and followed by liq-liq extraction. To the extracted residues were added IS and 50% CH₃CN for a total volume at 200 µl. The non-extracted control samples were blank cut hair samples without added drug that were processed in the same way as above. To the residues of extracted blank hair samples were then added drug at L, M and H concentration

levels, IS and 50% CH₃CN for a total volume at 200 µl. The extraction recovery was calculated as: peak area ratio of extraction sample/peak area ratio of non-extraction sample.

The extraction efficiency for incurred NVP hair samples was evaluated by measuring how much drug remained in the hair residues after repeated extractions. To the incurred NVP cut hair samples, we added 1 ml of MeOH/TFA (v/v, 9:1) and IS, and the samples were shaken at 37°C in a water bath overnight, followed by evaporation by N₂ and the liq-liq extraction procedures. The organic layer was then transferred to another tube, evaporated by N₂ and reconstituted with 200 µl of 50% CH₃CN, and this sample served as the 1st extraction sample. The aqueous sodium phosphate buffer (pH 9.4) layer was carefully discarded using a pipette, and to the hair residues were added 1 ml of MeOH/TFA (v/v, 9:1) and IS, followed by the same water bath at 37°C overnight, followed by evaporation by N₂ and liq-liq extraction procedures as described above. This sample was the 2nd extraction sample. The aqueous sodium phosphate buffer (pH 9.4) layer was then carefully discarded using a pipette, 1 ml of MeOH/H₂O (v/v, 8:2) and IS were added to the hair residues after the 2nd extraction, followed by the same water bath, evaporation by N₂ and liq-liq extraction procedures as described above. This sample then served as the 3rd extraction sample. All three extracted samples were analyzed by LC-MS/MS. The NVP levels in the 1st, 2nd and 3rd extractions were compared.

Effects of hair washing and UV light on hair NVP tests

Hair washing on NVP content in hair—Each incurred hair sample for two subjects was divided into two parts. One part was washed with shampoo (Pantene Pro-V, Fine Hair Solutions) for approximately three minutes, followed by warm water (around 40–45°C) rinse; hair conditioner was then added for two minutes, and the hair was washed again with warm water. The washed hair was then dried in the hood and cut to about 1–3 mm segments. The other part of the incurred hair sample from the same patient was left unwashed and directly cut into about 1–3 mm segments. About 2 mg of the washed hair and control samples were then analyzed for NVP content.

Ultraviolet (UV) light exposure of the hair sample on NVP content—The pooled incurred hair sample was divided into two parts; one part was directly exposed to the biological safety cabinet's UV light (253.7 nanometer region) for three days, then cut into about 1–3 mm segments, and the other part (the control) was put into foil to shield from UV-light exposure, then cut into about 1–3 mm segments. Both the UV-light exposed and control samples were measured for NVP content.

Clinical hair sample analysis

Small hair samples from HIV-positive participants on cART in the WIHS cohort were collected from the occipital portion of the scalp during semiannual visits. The hair collection process has been described previously [24]: briefly, hair samples were cut as close as to the scalp as possible, labeled on the distal end to denote directionality, and stored in tin foil at room temperature. The proximal section of the hair sample closest to the scalp (about 1 cm) is cut into 1–3 mm length segments with scissors and placed in a pre-weighted glass test tube. The tube is then re-weighted to obtain the exact sample weight of the cut hair specimen (~2 mg for each sample). Each cut hair samples is then mixed with 1 ml of MeOH/TFA (v/v, 9:1) and IS, and the samples are then processed and analyzed by LC-MS/MS in the same way as the calibration curve samples and QC samples described above.

Analysis of NVP in a single strand of hair

A single strand of a NVP incurred hair sample approximately 1.5 cm in length, was weighed on the Sartorius analytical balance (MC210P model), cut into several segments with small

surgical scissors and placed into a test tube. The single strand cut hair samples were then shaken with 1 ml of MeOH/TFA (v/v, 9:1) and extracted by the same methods as previously described. The extracted hair samples were reconstituted with 100 µl or 200 µl of 50% CH₃CN and 10 µl were injected into the LC-MS/MS.

Results and Discussion

Method Development

One challenge in developing a method for analyzing drugs in hair is in the extraction process. Since hair is a solid tissue and most of the drug is bound tightly to components of the hair (melanin /or keratin), while spiking a hair sample with drug does not completely mimic a true clinical hair sample. To test the efficiency of drug extraction, therefore, an incurred hair sample must be used. The typical process of hair sample preparation involves cutting hair into small segments, grinding hair to a powder or mechanically pulverizing the hair by a mill or a bead beater etc., prior to extraction by solvent(s) or enzyme digestion [16]. To test whether NVP could be efficiently extracted from cut hair as well as from powdered hair, we divided one patient's pooled hair sample (black color) into two parts. One part was cut into small segments with scissors; the other part was ground into powder via frozen mill. Both the cut hair and powdered hair samples were extracted in the same way with MeOH/TFA (v/v, 9:1) shaken at 37°C overnight, followed by liq-liq extraction for sample clean-up. As Figure 2 shows, similar NVP levels were found from these two kinds of hair sample processing methods, which indicates grinding hair to a powder is not likely to be necessary for efficient NVP extraction. Since grinding hair samples to powder is very time consuming and some of the hair sample may be lost in the grinding tube, a grinding hair method is likely not suitable for high through-put assays and when the hair sample quantities are limited.

Methanol is the most commonly used solvent for drug extraction of hair samples. To extract NVP efficiently from cut hair samples, we compared different solvents, including methanol, methanol/TFA (v/v, 9:1) and acetonitrile, for samples shaken at 37°C in a water bath overnight (~14 h). We found that methanol was close to methanol/TFA (v/v, 9:1) for NVP extraction, but the acetonitrile was less than 15% of methanol/TFA extraction (Electronic Supplementary Material Figure S-2). Aqueous methanol (50% MeOH or 90% MeOH) was also less efficient than methanol/TFA for NVP hair extraction (Electronic Supplementary Material Figure S-3). To further optimize the extraction conditions, the time-course of extraction of NVP by methanol/TFA from cut incurred hair samples was determined. As shown in Figure 3, a 14-hour extraction approximated the extraction rate out to 24 hours and was sufficient for maximum extraction. The optimal hair extraction conditions for NVP were therefore determined to be methanol/TFA (v/v, 9:1) with an overnight (>14h) incubation. As shown in Figure 4, the NVP in a hair sample is selectively detected.

Method Validation

Specificity—Six lots of blank hair samples from healthy volunteers were tested and no interference peaks were observed at the retention times of the drug or IS, which indicates the method is highly specific for NVP assay.

Linearity—A good linearity of the spiked drug concentrations from 0.25 to 100 ng/mg hair for NVP versus the drug to IS peak area ratio was obtained. The regression coefficients were more than 0.99. Using 2 mg cut hair samples, the limit of quantification (LOQ) was set at 0.25 ng/mg hair (S/N>20), which is sufficient to detect NVP in hair samples from patients on NVP.

Assay accuracy and precision—The intraday and interday accuracy and precision for spiked quality control (QC) samples are summarized in Table 1. The accuracies of low, medium and high QC concentrations for NVP were 98.3–105.9% with coefficients of variation (CV) less than 10%. Since spiked hair QC samples may not completely mimic incurred hair samples, a pooled cut hair sample from a patient receiving NVP therapy was aliquotted and analyzed on different days to test the assay reproducibility for clinical hair samples. As shown in Table 1, the CVs of inter- and intra-day incurred human hair QC samples were less than 10%. These results indicate that the established method has good precision for the measurement of NVP in incurred human hair samples.

Matrix effect—The matrix ionization suppression or enhancement of hair NVP was assessed by measuring the matrix factor (MF). The absolute MFs at the medium concentration from six lots of hair samples were from 0.88 to 1.08 (Table 2). The CVs of absolute MF and IS normalized MF from the six lots of hair samples were all <10%, which suggests that no significant matrix effect was found for the NVP hair assay.

Extraction Recovery—Since a spiked drug hair sample does not completely mimic incurred hair samples, the NVP extraction recovery from hair samples was evaluated in two ways: one from the spiked hair samples and the other from the incurred hair samples. The extraction recovery for the spiked drug hair samples in triplicate at low, medium and high concentrations were 92%, 89% and 94% respectively. The spiked hair sample extraction recovery more likely represents the liq-liq extraction recovery procedure rather than the first MeOH/TFA (v/v, 9:1) extraction step's recovery, because spiked NVP cannot completely enter into the intact hair tissue to mimic the incurred hair sample. To evaluate the extraction efficiency of the current method for analyzing NVP in clinical hair samples, we repeated extracting for the incurred hair samples three times to measure how much drug remains in the hair residue. As Figure S-4 shows, the 2nd MeOH/TFA extraction and the 3rd MeOH/H₂O extraction were less than 5% and 0.3% of the first MeOH/TFA extraction respectively, which indicates that at least 95% of NVP had been extracted by the first MeOH/TFA extraction step, leaving insignificant amounts of drug in the remaining hair residue.

Stability test—Pure NVP was stable in MeOH/TFA (v/v, 9:1) at 37°C overnight, which indicates that NVP did not degrade under hair sample extraction conditions. The incurred NVP hair QC sample stored at room temperature was found to be stable for at least two and half years. Hair is a dried tissue and lacks metabolizing enzymes, so most drugs can remain stable in hair tissue for prolonged periods of time if the sample is stored at relatively dry environment.

In order to test if regular hair washing would change the NVP hair level or not, we divided the same incurred NVP hair samples into two parts: one part was washed with a commercial shampoo, hair conditioner and water, then dried. The NVP level in the washed hair was analyzed and compared with that of control hair samples. As Figure S-5 shows, no significant change of NVP level was found in treated hair samples. This result indicates that regular hair washing, such as in a shower, may not significantly affect the hair NVP level and it also suggests that the NVP in hair is mainly located inside of the hair tissue and the amount of NVP at the hair surface which may come from sweat or sebum glands was limited. Since NVP is used orally only and is not a volatile drug, external contamination by NVP of scalp hair samples was considered very low. Therefore, decontaminating scalp hair samples, a procedure which is used widely when analyzing illicit substances in hair samples, seems not to be necessary when analyzing ARVs in hair.

UV-light exposure of NVP incurred hair samples for 3 days also did not change the NVP hair level significantly (Hair weight normalized peak area ratios were 6.7 ± 0.4 (mean $\pm$ SD),

n=3, for UV treated, and 6.1 ± 0.13 (n=3) for control, $p=0.11$ by t-test.), which suggests sunlight exposure of hair is not likely to affect hair NVP levels significantly.

Clinical hair sample analysis

This method has been successfully applied to analyze more than 1000 clinical hair samples from participants in the WIHS cohort [24–26]. The CVs of the spiked QC samples at L, M and H concentrations were 9.6%, 5.2% and 5.4%, respectively (n=56). The mean accuracies of L, M and H QC samples were 93.3%, 99.4% and 103.9%, respectively. The CV of incurred hair QC samples was 6.5% (n=56). This result indicates that this method is robust and has good reproducibility.

A single strand of hair sample analysis

NVP is a highly lipophilic drug and widely distributed into tissues [28]. The high NVP level found in human hair makes it possible to detect NVP even in a sample of a single strand of hair. The weight of a single strand of hair of 1.5 cm in length is approximately 0.1 mg. The median NVP level in the hair of HIV patients on NVP-based treatment is around 30 ng/mg. If we use a 0.1 mg hair sample, its concentration in 200 μ l of reconstitution solution should be $30 \text{ ng/mg} \times 0.1 \text{ mg} / 200 \text{ } \mu\text{l} = 15 \text{ ng/ml}$. Our current LC-MS/MS system using Micromass Quattro LC can detect NVP at 1 ng/ml (injection volume 10 μ l), (S/N>5). The detection limit can be further increased by a factor of four if we inject a larger volume (20 μ l) from a sample reconstituted with less volume (100 μ l). In addition, when we used a relative new model triple quadrupole equipment such as Micromass Ultima or Sciex API-5000, about 2–4 times higher sensitivity for NVP detection was achieved (data not shown). Theoretically, therefore, hair analysis of NVP using a single strand of hair should be possible. To investigate if we can analyze NVP in a single hair sample by our current method, single hair strands from several AIDS patients on NVP-based therapy were cut to about 1.5 cm in length and weighed, cut into several segments with scissors, extracted by MeOH/TFA as described above, and analyzed by LC-MS/MS. As Figure 5 shows, the NVP peak could be easily detected in a single hair sample. To test if hair weight affects the assay results or not, different weights of cut hair samples from one patient were analyzed under the same conditions. As Figure 6 shows, the drug to IS peak area ratio was linear ($r=0.99$) with hair weights from 0.1 mg to 4 mg. The intercept was relatively small and the peak area ratio/hair weight was very consistent, which indicates that hair weight does not affect the accuracy of the assay.

Since hair has different growth cycles, the drug concentration in each hair strand may not be homogeneous. To investigate whether the hair drug level in a hair sample is homogeneous or not, six hair strand samples from one patient's occipital scalp area hair were cut to about the same length and weighed (0.10–0.13 mg), then cut into several small segments, and analyzed for NVP. The CVs of the peak area ratio and peak area ratio/hair weight from the six hair strands were 12.7% and 13.7%, respectively, which indicates that the drug concentration in each hair strands from the occipital scalp region was relatively homogeneous. This experiment also suggests that, even with single strand hair samples, our assay can achieve a satisfactory precision.

Single strand hair sample analysis has several implications: 1). To investigate the homogeneity of a drug or endogenous biomarker in human hair samples; 2). To study the mechanism of drug incorporation into human hair from the systemic circulation; 3). To study drug use history by hair segmental analysis; and 4) A single hair strand sample is relatively clean and has a lower matrix effect. A single strand hair sample has been used for DNA analyses in the field of forensics. Here we show that a single strand hair sample can be used to identify if a patient has been adherent to the NVP in their cART regimen or not.

Recently, the dried blood spot (DBS) technique has become a hot topic in the drug bioanalytic field [36, 37] because this technique allows for analysis of drug in blood using just one small drop, which is usually around 5–50 μ l. Assuming blood density is 1 g/ml, 5 μ l of blood is ~ 5 mg. A single strand of hair at 1.5 cm length is only 0.1 mg, which is equivalent to 0.1 μ l of blood weight. The reason that NVP is more easily detected in hair than in blood plasma is due to the fact that NVP levels in hair are 5–10 times higher than that in plasma. The NVP median level in HIV patients on NVP based treatment is around 30 ng/mg (or 30 μ g/g), while the steady state NVP level in plasma is around 3–6 μ g/ml (or μ g/g) [30, 31]. NVP, therefore, is more concentrated in hair tissue than in plasma. Compared with DBS, hair sampling is non-invasive and more acceptable to patients. Also, there is no special training required for collecting hair samples (as compared to collecting blood samples) and no needles are involved in collecting hair, which can minimize the risk of needlestick injury or occupational HIV transmission events.

Conclusions

A sensitive, specific and reproducible LC/MS/MS method has been developed and validated for measuring the ARV drug, nevirapine, content in human hair in very small hair samples. This method will allow us to monitor NVP's long-term drug exposure in HIV infected patients using even a single strand of human hair. Hair drug analysis provides an alternative approach to conventional blood drug bioanalysis for assessing drug exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(A)

(B)

Figure 1.
Product ion mass spectra of NVP (A) and NVP-d5 (B)

Figure 2.

Comparison of the extraction of nevirapine from cut hair and powdered hair samples. One pooled hair sample was divided into two parts. One part was cut into small segments with scissors; the other part was ground into powder via frozen mill. Both the cut hair and powdered hair samples were extracted in the same way with MeOH/TFA (v/v, 9:1) shaken at 37°C overnight, followed by liq-liq extraction and analyzed by LC-MS/MS. Data are represented by mean±SD (n=3).

Figure 3.

Time course for extraction of nevirapine from cut hair incurred samples in MeOH/TFA (9/1) at 37°C. Each point represents mean±SD (n=4).

Figure 4.

LC-MS/MS chromatograms of hair nevirapine analysis. A), blank hair; B), blank hair spiked with IS; C), blank hair spiked with IS and nevirapine at LOQ concentration; D), clinical hair sample from a patient on nevirapine treatment.

Figure 5.

A single strand of hair nevirapine analysis. A single strand of nevirapine incurred hair sample at 1.5 mm length was cut into several segments with scissors, extracted by MeOH/TFA (9/1) at 37°C overnight, followed by liq-liq extraction and analyzed by LC-MS/MS.

Figure 6.

Linearity of nevirapine peak area/IS ratio with the hair sample weight. Different weights (0.01 mg to 4 mg) of the one pooled incurred hair sample were measured under the same conditions.

Table 1

Accuracy and precision for spiked QC samples and incurred QC samples

Drug	Sample Type				Spiked QC						Incurred hair QC samples*	
					Intraday			Interday			Intraday	Interday
	Concentration/Patient ID	LOQ	L	M	H	LOQ		L	M	H	A	A
	Spiked Conc. (ng/mg hair)	0.250	0.750	5.00	70.0	0.250		0.750	5.00	70.0		
NVP	Measured Conc. (ng/mg hair)	0.261	0.794	5.27	70.5	0.261		0.789	5.20	68.8	15.9	15.3
	Accuracy(%)	104	106	105	101	104		105	104	98.3		
	CV(%)	9.6	7.1	5.4	2.9	11		4.4	4.8	2.6	2.6	3.1
	n	6	6	6	6	6		12	12	12	6	12

* Incurred hair QC samples were prepared from a patient receiving NVP treatment.

Table 2

Matrix factor for NVP hair assay

Hair matrix	Absolute Matrix Factor (MF)	IS Normalized Matrix Factor
Lot Blank-A	0.89	0.98
Lot Blank-B	0.88	0.99
Lot Blank-C	0.96	0.95
Lot Blank-D	1.08	1.01
Lot Blank-E	0.93	1.00
Lot Blank-F	0.91	0.99
Mean	0.94	0.99
SD	0.074	0.022
CV(%)	7.9	2.3

The matrix factor was measured for each lot of hair samples at medium concentration in duplicate.

Absolute MF= (the analyte peak area in hair matrix)/(the analyte peak area in control without hair matrix).

IS normalized MF= (the analyte vs IS peak area ratio in hair matrix)/(the analyte vs IS peak area ratio in control without hair matrix).