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A validated liquid chromatography tandem mass spectrometry method for the analysis of efavirenz in 0.2 mg hair samples from HIV-infected patients

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Abstract

RATIONALE—Drug levels in hair provide a longer window of detection, compared to plasma drug levels, and therefore hair analysis has the advantage of assessing adherence over a longer period of time. No methods for the analysis of antiretroviral drugs in hair currently exist in South Africa, and worldwide there is only one validated method for the determination of efavirenz in hair that has been published.

METHODS—Efavirenz was extracted from 0.2 mg of hair through a simultaneous pulverization and extraction step. Separation was achieved on an Agilent Poroshell C18 column using an isocratic elution with a total run time of 3 min. A triple quadrupole mass spectrometer set to electrospray ionization in positive multiple reaction monitoring mode was used for detection. The method was validated over the concentration range 0.625 – 40 ng/mg.

RESULTS—Using ten times less hair than in a previously published method, the LLOQ was validated at 0.625 ng/mg. The interday and intraday assay precision, expressed as the percentage coefficient of variation (CV), for spiked calibration standards and quality control samples were lower than 7% and accuracy ranged from 97% to 110%. For quality controls prepared from authentic hair the % CV was less than 12%. The extraction efficiency for authentic QC samples was determined to be 83% after repeated extractions of the same samples.

CONCLUSIONS—This paper reports the first quantitative method for the determination of efavirenz in hair to be developed in South Africa. The validated method allowed for the successful monitoring of efavirenz in hair collected from HIV-infected patients as part of a clinical study.

Keywords

hair; efavirenz; LC/MS/MS; adherence; TDM

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Introduction

Efavirenz (EFV) is a non-nucleoside reverse-transcriptase inhibitor (NNRTI) used for the treatment of HIV infection worldwide ^[1], and is an important component of highly active antiretroviral therapy (HAART). EFV has a long half-life of 52 to 76 hours following a single oral dose, and 40 to 55 hours after multiple doses. As a result of its long half-life EFV is administered once a day as a single 600 mg dose ^[2]. In South Africa, the most effective antiretroviral therapy (ART) combination includes EFV in combination with tenofovir and either emtricitabine or lamivudine ^[3].

Therapeutic drug monitoring (TDM) plays an important role in monitoring a patient's response to ART by measuring antiretroviral (ARV) drug levels. Sub-therapeutic levels of EFV can put patients at risk for treatment failure as well as for the development of drug resistance, whereas super-therapeutic EFV levels have been associated with CNS side effects ^[4]. It is therefore important for successful response to ART to ensure that drug levels are maintained within therapeutic ranges. One of the most common reasons for sub-therapeutic levels of ARV drugs is poor adherence ^[5]. Current measures used to measure adherence include patient self-report, pill counts, prescription refills and electronic medication monitors ^[6]. However, each of these methods comes with limitations. Self-report measures, for example, often overestimate adherence ^[7].

A more direct method of measuring adherence is by measuring drug exposure as is performed in TDM. Conventionally, drug exposure is measured by determining concentrations of a drug, or its metabolite, in plasma ^[6]. However, plasma concentrations usually only provide a short-term (hours to 1 to 2 days) assessment of drug exposure and therefore cannot predict long-term treatment outcomes ^[8]. Plasma drug concentrations are also susceptible to 'white coat effects' where patient adherence improves just prior to clinic visits ^[9]. Therefore, a single drug concentration in plasma might not reflect an accurate measure of actual drug exposure ^[10].

More recently, hair has been studied as an alternative matrix for TDM. Drug levels in hair provide a longer window of detection (weeks to months) and therefore hair analysis has the advantage of assessing long-term adherence ^[8] and provides an estimation of the average level of drug exposure over time ^[11]. Concentrations of ARVs in hair have been shown to be closely correlated with virologic outcomes ^[12–20].

As a result of the potential that hair analysis holds for TDM and adherence studies, LC/MS/MS methods have, in recent years, been developed for the analysis of ARVs in hair. Studies have reported sensitive LC/MS/MS methods for the analysis of the ARVs, EFV, lopinavir and ritonavir ^[21], nevirapine ^[10], as well as abacavir and tenofovir ^[22] in hair. To date, only one validated method has been published for the analysis of EFV in hair ^[21]. This method, published in 2008 by Huang *et al.* ^[21], was validated over the range of 0.05 – 20 ng/mg using 2 mg of hair.

The Huang *et al.* ^[21] method has been used for the determination of EFV levels in hair in the context of other studies ^[11,18,23,24], however, the amount of published data regarding EFV levels in hair remains limited. Moreover, there is only one study that reports EFV levels in

hair samples collected from South Africans [24]. In this study hair samples (n = 134) collected from HIV-infected South African females were sent to an international laboratory for the analysis and quantification of EFV. The median EFV concentration in the hair samples was reported to be 5.60 ng/mg (IQR: 3.93 – 7.06 ng/mg). In this case, EFV levels in hair were not found to be associated with virologic response [24].

There is a need for the development of validated methods for the analysis of ARVs in hair in South Africa to compliment current methods of TDM and to provide objective measures of assessing long-term adherence. Additionally, it is also possible to improve on some aspects of the method published by Huang *et al.*[21] in 2008. This paper reports the first LC/MS/MS method for the quantitative determination of EFV to be developed and validated in South Africa. The validated method was used to measure EFV levels in clinical hair samples within the context of adherence monitoring.

Experimental

Collection and storage of hair samples

EFV free hair, collected from a healthy child, was used during method development, preparation of calibration standards and quality controls (QCs) and validation experiments. Seven additional sources of EFV free hair for matrix effects and specificity experiments were collected from healthy adult volunteers. Hair positive for EFV was collected from an adherent patient on an EFV-based ART regimen and was used during method development experiments and throughout the study as an authentic QC.

Chemicals

EFV and its deuterated analogue (EFV-d5), used as an internal standard, were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Stock solutions of 1 mg/ml were prepared in methanol (for EFV) and acetonitrile (for EFV-d5). Working solutions were prepared by diluting EFV and the internal standard to 10 and 1 µg/ml with methanol. All stock and working solutions were stored at ~ -80°C. Methanol (gradient grade) was purchased from Merck (Darmstadt, Germany) and acetonitrile (LC-MS grade) was purchased from Honeywell International Inc. (Mexico City, Mexico). Formic acid (~ 98%) was purchased from Fluka Analytical (Buchs, Switzerland). Deionized water was prepared using a Synergy Water Purification System purchased from Merck Millipore (Massachusetts, USA).

EFV extraction from human hair

For the extraction of EFV, 0.2 mg of hair was weighed into 2 ml screw-cap tubes to which two metal balls (2.4 mm) were added. The metal balls and screw-cap tubes were purchased from Omni International Inc. (Georgia, USA). Extraction solvent (250 µl) was added to each tube before pulverizing the hair samples. The extraction solvent was made up of a mixture of methanol and water (70:30, v/v) with the internal standard added at a concentration of 12.5 ng/mg. Samples were vortex mixed briefly for 10 s and pulverized using the Omni Bead Ruptor 24 (Omni International Inc., Georgia, USA) at a speed of 5 m.s⁻¹ for five cycles of 60 s each, with a dwell time of 30 s after each cycle. After the samples were pulverized, the

extract was removed and filtered through a 0.45 µm filter under vacuum (Merck Millipore, Massachusetts, USA). The clear extract (10 µl) was injected into the LC/MS/MS system for analysis.

Liquid chromatography/tandem mass spectrometry

EFV was analysed using an Agilent Technologies 1260 Infinity Liquid Chromatography system (California, USA) coupled to an AB SCIEX Q Trap 5500 mass spectrometer (SCIEX, Massachusetts, USA). Chromatographic separation was achieved using an Agilent Poroshell C18 column (2.7 µm, 4.6 × 50 mm) (Agilent Technologies, California, USA) kept at 30°C. The system was run using an isocratic elution with mobile phase made up of 0.1% formic acid in water and acetonitrile (20:80, v/v). The total run time was 3 min at a constant flow of 500 µl/min.

The mass spectrometer was set to electrospray ionization in the positive multiple reaction monitoring mode (MRM). The mass spectra for EFV and EFV-d5, shown in Figure 1, were obtained by direct infusion of each of the reference standards into the mass spectrometer. EFV was monitored using two transitions, 316 → 244 (quantifier ion) and 316 → 232 (qualifier ion). EFV-d5 was monitored at 321 → 246. Flow injection analysis was used to optimize the mass spectrometer source parameters for the analysis of EFV. The ion spray voltage was set at 5500 V with a source temperature of 500°C. The nebulization, heating and curtain gases were set to 60, 60 and 30 psi, respectively. The MRM transitions and final mass spectrometer conditions are described in Table 1. Analyst software version 1.6.2 (SCIEX, Massachusetts, USA) was used to analyse the data collected.

Method validation

The method was validated according to the international guidelines of the Food and Drug Administration (FDA) [25,26] as well as the European Medicines Evaluation Agency (EMA) [27,28].

Standard curve and quality control samples—EFV free hair was used for the preparation of the calibration standards and QCs. The preparation of the calibration standards and QCs was based on the extraction method developed for authentic hair samples as previously described, however, the order of the steps were altered slightly to reduce the time taken to prepare the spiked standards and QCs. Additionally, blank hair was extracted in larger quantities to simplify the process; 0.8 mg rather than 0.2 mg of hair was extracted in each screw-cap tube. Extraction solvent (1 ml) with 12.5 ng/mg internal standard was added to 0.8 mg blank hair in screw-cap tubes containing two metal balls. The hair samples with extraction solvent were vortex mixed briefly for 10 s and pulverized accordingly. The extracted solvent from each tube was removed and pooled together in a separate, larger tube.

The extracted solvent was used to prepare the calibration standards and QCs. For the calibration standards EFV was spiked into extracted solvent at a concentration of 32 ng/ml and serially diluted 1:1 with extracted solvent to produce concentrations of 16, 8.0, 4.0, 2.0, 1.0 and 0.5 ng/ml (corresponding to 40, 20, 10, 5.0, 2.5, 1.25 and 0.625 ng/mg). For the preparation of the high, medium and low QCs as well as the lower limit of quantitation

(LLOQ), EFV was spiked into extracted solvent at a concentration of 26 ng/ml (high QC) and diluted with extracted solvent to produce concentrations of 13 ng/ml (medium QC), 1.25 ng/ml (low QC) and 0.5 ng/ml (LLOQ) (corresponding to high, medium, low QCs and the LLOQ at 32.5, 16.25, 1.56 and 0.625 ng/mg respectively). The calibration standards and QCs were kept at $\sim -80^{\circ}\text{C}$ until required. For each of the three validation batches, the calibration standards were analysed in duplicate and the QCs in six-fold. Before injection into the LC-MS/MS system, the standard and QC solutions were filtered as previously described.

Aliquots from a homogenous sample of authentic hair, positive for EFV, were weighed into screw-cap tubes (0.2 mg) and extracted using the extraction method described. Authentic QCs were included for analysis in each of the validation and sample batches to monitor the reproducibility of the extraction procedure.

Stock solution stability—Measurements for the comparison of solutions for the determination of short and long-term stock solution stability were made using an Agilent 8453 ultraviolet (UV) - visible Spectroscopy System (Agilent Technologies, California, USA). Measurements were taken at two fixed wavelengths of 204 and 247 nm. A stock solution (1 mg/ml) of EFV was prepared in methanol. Aliquots of the solution (test solutions) were kept at room temperature ($\sim 22^{\circ}\text{C}$), $\sim 4^{\circ}\text{C}$ and $\sim -20^{\circ}\text{C}$ for 72 hours. The absorbance of each test solution and the reference solution (kept at $\sim -80^{\circ}\text{C}$), prepared in triplicate at 10 $\mu\text{g/ml}$ with a mixture of methanol and water (70:30, v/v), was measured and compared. Long-term stock solution stability (61 days at $\sim -80^{\circ}\text{C}$) was determined by comparing the stock solution to a fresh stock solution prepared on the day of stability testing using UV-visible spectrophotometry as described above.

On-instrument stability—High, low and authentic QCs extracted in the first validation batch were left in the autosampler (kept at 4°C) and re-injected after approximately 24 and 48 hours in order to evaluate the stability of EFV in processed samples. The peak area ratios of the re-injected QCs were compared to the peak area ratios of the QCs from the first injection.

Matrix effect—The method described by Matuszewski and co-workers [29,30] was followed to study possible ion enhancement or suppression effects that could be caused by co-eluting matrix components on the ionisation of EFV. EFV blank hair obtained from eight healthy volunteers was spiked with EFV at high, medium and low QC concentration levels (32.5, 16.25, 1.56 ng/mg) and a single concentration of internal standard. The peak area ratios of the analyte/internal standard at each concentration level for each of the sources of blank hair were used to generate regressions for each individual matrix.

Extraction recovery—The repeated extraction of authentic hair samples was used to investigate the efficiency of the extraction method. Authentic hair was extracted, in six-fold, using the extraction method developed. The extract was removed for analysis and the hair was left to air dry at room temperature. This extraction process was repeated on the authentic hair a further two times. The extracts from each of the extractions were filtered and

analysed in order to compare the amount of EFV that remained in the hair after each of the extractions.

Specificity—Specificity for EFV was evaluated by analysing blank hair samples collected from six healthy volunteers for possible interference between EFV and endogenous substances. A significant interference peak was defined as a peak at the EFV retention time with a peak area greater than 20% of the peak area of the LLOQ.

Carry-over—Carry-over was assessed and monitored during all of the analytical runs by injecting a double blank sample (containing no analyte or internal standard) immediately after the highest calibration standard

Clinical hair sample analysis

Hair samples were collected from 135 HIV-infected patients (9 males and 126 females) who were enrolled within a larger randomized controlled trial [31]. Patients recruited for the study commenced ART at a treatment centre in Gugulethu, Cape Town. A total of 257 hair samples were collected during three visits to the centre at weeks 16 (n = 93), 32 (n = 75) and 48 (n = 89). Hair samples were cut from the posterior vertex region of the head as close to the scalp as possible and placed into paper envelopes. Proximal and distal segments of the hair were clearly marked. Hair samples were stored at room temperature until analysis. The hair samples were quantitatively analysed using the validated method described above. Small samples of hair (~ 0.2 mg) were cut from the proximal section of each of the collected hair samples (about 1 cm) with scissors, placed in 2 ml screw-cap tubes and weighed. The mass of the tube was subtracted from the total mass in order to get the exact mass of the hair sample, which was recorded. The hair samples were extracted and analysed by LC/MS/MS using the methods described above.

Ethical Approval

The University of Cape Town Faculty of Health Science Research Ethics Committee gave approval to conduct this study (HREC/REF: 101/2015 and 387/2015). Each participant provided written informed consent.

Results

Extraction method

A simple and quick method was developed and optimized for the extraction of EFV from only 0.2 mg hair samples. Hair samples were not decontaminated prior to extraction. Since EFV is taken orally external contamination of hair by EFV was not a concern. The simultaneous pulverization and extraction procedure developed allows for up to 24 hair samples to be processed, within 10 min, at a time.

Method validation

The extraction method for EFV from hair performed well during validation. The calibration range was validated between 0.625 – 40 ng/mg and a quadratic regression, with peak area ratio (analyte/internal standard) against concentration with $1/(\text{concentration} \times \text{concentration})$

($1/x^2$) weighting, was fitted to the curves. Each of the calibration curves showed a good fit, with all regression coefficients (R^2) above 0.98. The intraday and interday accuracy and precision (CV %) for the spiked QCs and the precision for the authentic QCs are summarised in Table 2. The accuracies of the spiked QCs were between 101.1 and 107.9% with CVs below 7%. The intraday and interday CVs for the authentic QC samples were below 12%.

Short-term stock solution stability was demonstrated for up to 72 hours at room temperature ($\sim 22^\circ\text{C}$), $\sim 4^\circ\text{C}$ and $\sim -20^\circ\text{C}$. The % difference between the absorbance of the reference and test solutions kept at each of the described conditions was less than 4% at $\lambda = 204\text{ nm}$ and less than 7% at $\lambda = 247\text{ nm}$ with CVs below 1%. Long-term stock solution stability was demonstrated at $\sim -80^\circ\text{C}$ for at least 61 days. At $\lambda = 204\text{ nm}$ the % difference between the absorbance of the reference and test solution was 8.7% and at $\lambda = 247\text{ nm}$ the % difference was 4.4% with CVs below 1%.

On-instrument stability was demonstrated for both high and low spiked QCs as well as authentic QCs for up to 48 hours. The % difference between the first QC injections and the re-injected QCs was less than 1.5% for the spiked and authentic QCs after 24 and 48 hour re-injections (CV % < 10). Therefore, samples processed for EFV are stable in the autosampler for up to 48 hours.

Eight sources of blank hair were used to assess matrix effects. The slope variability was found to be 2.0%. The CV of the eight peak area ratios (drug/internal standard) was 1.6, 1.2 and 11.5% at the high, medium and low concentration levels respectively (Table 3). This result indicates that the precision of this method is not affected by matrix effects and that sample analysis from different sources is both reliable and reproducible.

Since spiking hair with drugs does not mimic the processes whereby drugs are incorporated into hair, the extraction efficiency of the method was determined by investigating how much EFV remained in samples of authentic hair after extraction, as was done in a previous study [21]. Authentic hair was extracted three times and the amount of EFV removed by each extraction was measured (Figure 2). The first extraction removed approximately 83% of EFV incorporated into the authentic hair.

No significant interference peaks were observed for EFV in any of the six blank hair samples analysed indicating that this method is highly specific for the analysis of EFV. The signal to noise ratio at LLOQ (0.625 ng/mg) was above 20, which is sufficient to detect EFV in samples of hair from patients on EFV. The accuracy for the LLOQ (N=18) was 106.5% (CV% = 6.6). No carry-over was observed. LC/MS/MS chromatograms for the analysis of EFV in hair are shown in Figure 3.

Clinical hair sample analysis

The validated method for the quantification of EFV in hair was used to analyse the hair samples (n = 257) collected for the broader clinical study in order to monitor the patients EFV levels in hair over the 48 week study period. The median EFV levels at weeks 16, 32 and 48 were 5.44 ng/mg (IQR: 3.60 – 9.72), 5.63 ng/mg (IQR: 2.72 – 8.17) and 4.80 ng/mg

(IQR: 2.65 – 7.93) respectively. The mean EFV level for the duration of the study period was 7.40 ng/mg (± 7.58). The significance of these results in the context of the broader study will be discussed elsewhere.

Discussion

The simple and quick extraction method developed in this study is effective in removing EFV from very small samples of hair. The method involves pulverizing samples of hair into a fine powder. Pulverizing hair has previously been reported to increase the speed and simplicity of hair analysis for certain drugs and metabolites, such as ethyl glucuronide [32,33]. However, previous studies have found that pulverizing hair samples prior to the extraction of ARVs did not influence the amount of drug extracted, and comparable amounts of drug were extracted using hair samples cut into small segments [10,21]. In one of these studies cutting samples of hair was preferred to pulverizing or grinding hair into a fine powder. The main reason for this being that the type of grinding procedure used involved freezing hair samples with liquid nitrogen before grinding, which was a time consuming step [21]. The pulverization process used in this study, however, was a simple and quick process. Additionally, it was found that when working with very small amounts of hair, pulverizing hair samples was easier than cutting hair into small segments.

Due to the limited amount of hair available for the analysis of the clinical samples, this method had to be developed using very small amounts of hair. EFV is a lipophilic drug that accumulates well in hair due to its basic properties [34], and therefore high levels of EFV are found in hair making it possible to detect in even small amounts of hair. In a previously published method EFV was extracted from 2 mg of hair [21]. In the current method 0.2 mg of hair was used. Using 0.2 mg of hair the LLOQ was set at 0.625 ng/mg. Although a lower LLOQ could have been validated for this method, the sensitivity of the validated method is sufficient to determine EFV levels in samples of hair collected from HIV-infected patients currently on an EFV-based ART regimen.

The method was successfully validated over the described calibration range. In addition to spiked QCs, authentic QCs, which are more representative of clinical hair samples, were included in the validation batches. The precision of the authentic QCs was less than 12%, indicating that this method has good reproducibility in measuring EFV concentrations in authentic samples of human hair. Despite a simple and quick extraction method, results indicated that approximately 83% of EFV incorporated into authentic hair was removed during extraction. In calculating this result it was assumed that all of the EFV was removed after the third extraction. However, the results show that it is likely that a small amount of EFV still remained in the hair and that a fourth extraction might have been necessary to completely remove all of the EFV from the hair. Nonetheless, the results from this experiment indicate that the simple extraction method was effective at removing EFV from a small amount of hair in a short period of time, without the need for a long incubation step.

The validated method was successfully applied to 257 hair samples collected from HIV-infected patients. The concentrations of EFV found in the hair samples are comparable to EFV concentrations reported in previous studies [11,18,23,24]. Whilst these results are

significant as they provide insight into EFV levels measurable in hair, the therapeutic range for EFV in hair within the South African context needs to be determined in order to give quantitative results more value within the field of adherence monitoring.

In conclusion, this paper describes the validation and application of a sensitive and specific method for the quantification of the ARV EFV in 0.2 mg hair samples using LC/MS/MS techniques. One of the advantages of this method is the quick analysis time compared to the previously published method ^[21] which necessitated an overnight incubation step. The extraction and LC/MS/MS analysis of EFV using the method reported here is not time consuming and results can be obtained within a short period of time, which makes this method applicable for use within routine drug monitoring. Another advantage of this method is that only a very small amount of hair, 0.2 mg, is required for the extraction process. This is especially beneficial in the African context where it is often the case that limited quantities of hair are available for collection. This is the first method to be developed in South Africa for the monitoring of ARV drug exposure using hair as a matrix, and provides an alternative approach to conventional TDM methods currently in use within the country.

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Figure 1. Initial product scan mass spectra of (A.) efavirenz and (B.) efavirenz - d5. The proposed structures of the fragmented efavirenz ions are shown in A.

Figure 2. Percentage efavirenz extracted from authentic hair after three repeated extractions, where the total amount of efavirenz extracted was calculated by adding the amount of efavirenz extracted after each of the three extractions. Error bars show the standard deviation from the mean value (n = 6).

Figure 3.

LC/MS/MS chromatograms for the analysis of efavirenz in hair: (A.) blank (efavirenz free) hair; (B.) blank hair spiked with efavirenz at the LLOQ (0.625 ng/mg); (C.) authentic hair sample from an adherence patient on an efavirenz based ART regimen (6.87 ng/mg).

MRM transitions and final mass spectrometer conditions for the analysis of efavirenz and efavirenz-d5

Table 1

Analyte	Transition	Dwell Time (ms)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
EFV	316 → 244	200	81	10	19	20
	316 → 232	200	81	10	21	18
EFV-d5	321 → 246	200	86	10	17	24

Table 2
Accuracy and precision for spiked QC hair samples and authentic QC hair samples

	Spiked QCs						Authentic QCs	
	Intraday			Interday			Intraday	Interday
	H	M	L	LLOQ	H	M	L	LLOQ
Nominal concentration (ng/mg)	32.5	16.3	1.56	0.625	32.5	16.3	1.56	0.625
Measured concentration (ng/mg)	33.3	16.6	1.66	0.674	33.2	16.4	1.62	0.665
CV (%)	0.6	0.9	1.9	2.5	1.4	1.2	3.2	6.6
% Accuracy	102.5	101.9	106.6	107.9	102.3	101.1	103.9	106.5
n	6	6	6	6	18	18	18	5
								15

Table 3

Assessment of matrix effects of eight different hair matrices

	High 32.5 ng/mg	Medium 16.3 ng/mg	Low 1.56 ng/mg	Area Ratio vs Concentration Regression Slope
Mean ratio of peak areas	8.50	3.97	0.369	0.263
STDEV	0.140	0.0461	0.0422	0.00528
CV (%)	1.6	1.2	11.5	2.0