Determination of salivary efavirenz by liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

A novel and robust screening method for the determination of the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFV), in human saliva has been developed and validated based on high performance liquid chromatography tandem mass spectrometry (LC–MS/MS). Sample preparation of the saliva involved solid-phase extraction (SPE) on C18 cartridges. The analytes were separated by high performance liquid chromatography (Phenomenex Kinetex C18, 150 mm × 3 mm internal diameter, 2.6 μm particle size) and detected with tandem mass spectrometry in electrospray positive ionization mode with multiple reaction monitoring. Gradient elution with increasing methanol (MeOH) concentration was used to elute the analytes, at a flow-rate of 0.4 mL/min. The total run time was 8.4 min and the retention times for the internal standard (reserpine) was 5.4 min and for EFV was 6.5 min. The calibration curves showed linearity (r^2, 0.989–0.992) over the concentration range of 3.125–100 μg/L. Mean intra- and inter-assay relative standard deviation, accuracy, mean extraction recovery, limit of detection (LOD) and limit of quantification (LOQ) were 0.46–9.43%, 80–120%, 60% (±7.95), 1.84 and 6.11 μg/L respectively. The working range was defined as 6.25–100 μg/L. This novel LC–MS/MS assay is suitable for reliable detection of low EFV concentrations in saliva and can be used as a screening tool for monitoring EFV compliance.

1. Introduction

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor, used in combination therapy for HIV-1-infected children [1]. Marzolini and co-workers proposed a 1000–4000 μg/L plasma range at mid-dosing interval as a suitable target for EFV drug levels [2]. When EFV levels reach toxic levels (>4000 μg/L) a higher incidence of side-effects occurs such as insomnia, dizziness, abnormal dreams and loss of concentration [2,3], while subtherapeutic levels (<1000 μg/L) can lead to treatment failure due to viral resistance [4,5]. It is thus important to ensure that EFV plasma levels are within the therapeutic window.

Therapeutic drug monitoring (TDM) of antiretroviral (ARV) plasma concentrations can be useful in the optimization of HIV treatment [6,7], however, the collection of plasma is invasive, causes discomfort and is painful [8] especially in young children,

opposed to the collection of saliva which can be done by non-invasive, painless methods and has a diminished risk of HIV transfer to the health care worker [8]. However, for the use of saliva for TDM, a confirmed relationship should exist between the concentrations of the drug in the different body fluid matrixes [8]. An obstacle for the determination of EFV in saliva, is the protein binding of EFV, which is greater than 99% [1]. Saliva usually represents the free drug concentration of a drug [9], thus the salivary concentrations are very low (<1% of plasma levels). Methods to determine salivary levels of nevirapine [8,10] and indinavir [11] were previously reported, but none yet for EFV. To further investigate the possibility of assaying salivary EFV to monitor adherence or for therapeutic drug monitoring of EFV, a validated analytical LC–MS/MS based method for the analysis of EFV in saliva, was developed.

2. Materials and methods

2.1. Chemicals

Solvents used as eluents were high purity water and HPLC grade methanol (MeOH) (Burdick and Jackson Laboratory Co.); acetic acid (SAARCHEM) and ammonium formate (Agilent Technologies). EFV for the reference standards was supplied by the

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Table 1
Mass spectrometry parameters.

Analyte	MRM	Collision energy voltage (V)	Fragmentation voltage (V)
EFV	$316 \rightarrow 244$[a]	10	120
EFV	$316 \rightarrow 232$[b]	15	120
Reserpine (IS)	$609 \rightarrow 195$	45	200

a Transition used in the quantification.
b Transition of qualifier.

World Health Organization: International Chemical Reference Substances (Batch number 104229). Reserpine, the internal standard (IS), was acquired from Fluka (Batch number 1325032). All chemicals were stored at the appropriate storage temperatures and conditions.

2.2. Liquid chromatography/tandem mass spectrometry

2.2.1. Chromatographic systems

The chromatographic system consisted of an Agilent G1312A binary pump, a G1379B micro-vacuum degasser and a thermostated auto-sampler fitted with a six port injection valve and 100 μL capillary loop. EFV was separated from the internal standard on a C18 column (Phenomenex Kinetex C18; 150×3 mm internal diameter; 2.6 μm particle size). The temperature of the column was maintained at 40 °C. EFV in the eluent was detected with an Agilent 6410 triple quadrupole mass spectrometer using electrospray ionization (ESI) in positive mode. The most abundant fragment for each compound was selected by performing enhanced product ion scans of the standards during an infusion analysis. The selected multiple reaction monitoring (MRM) transitions are reflected in Table 1. The collision energy voltage, fragmentation voltage and capillary voltage were adjusted to provide the highest sensitivity, refer to Table 1. Nitrogen was used as nebulizer gas and desolvation gas. The gas temperature (°C), gas flow (L/min), capillary voltage (V) and nebulizer pressure (kPa) were set at 300, 12, 4000 and 275.8 respectively.

Agilent Technologies MassHunter Workstation Software, Version B02.00, 2008, was used for system control, data collection and quantitative analysis.

2.2.2. Mobile phases

A mobile phase gradient pumped at 0.4 mL/min was used to elute the analytes from the column. Mobile phase A consisted of methanol–water (10:90 (v/v)). Mobile phase B consisted of a 5 mM ammonium formate buffer in 97% MeOH, pH adjusted to 5.5 with acetic acid. The elution started at 10% B held isocratically for 0.6 min increasing to 100% B at 1.5 min and returning to 10% B at 8.2 min. The eluent was diverted from the mass spectrometer for the first 3 min and after 8.4 min to reduce contamination of the electrospray source. The retention times of the IS and EFV were 5.4 and 6.5 min respectively. Re-equilibration was performed during a 4 min postrun time.

2.3. Preparation of standards

A stock solution of EFV was prepared by dissolving 5 mg EFV in 5.0 mL MeOH/water (1:1). The solution was stored at −20 °C. IS stock solution was prepared by dissolving 2.95 mg reserpine in 100 mL MeOH and kept for a maximum of 1 month at −20 °C.

Working solutions were prepared by diluting the stock solution of EFV to a final concentration of 100 mg/L, and the IS to 5.96 μg/L.

Spiked saliva calibrants ($n = 6$) were prepared by serial dilution of the working solution over a concentration range of 3.125–100 μg/L, with blank saliva donated by a healthy drug naïve individual by chewing a Salivette® swab and then centrifuging the Salivette® for 10 min at $1500 \times g$ in the supplied recovery tubes.

Quality control (QC) samples ($n = 4$) were prepared at different concentrations (6.25, 12.5, 25, and 50 μg/L EFV), by spiking saliva with a separately made working solution.

2.4. Samples

2.4.1. Sample collection

Saliva samples were collected from HIV-infected children already on EFV based treatment regimens by sampling with a Salivette® swab that was chewed for 2 min and then placed into the supplied collection tube and centrifuged for 10 min at $1500 \times g$. Saliva samples were then collected in Eppendorf® centrifuge tubes and stored at −80 °C until further analysis. The exact time of saliva collection was recorded. Any food or drinks, except water were prohibited to be taken 30 min prior to saliva collection.

2.4.2. Sample preparation

Samples, calibrants and QC's were thawed at room temperature on the day of analysis. Aliquots of 100 μL saliva, calibrants or QC's were mixed with 20 μL of IS working solution, vortexed for 10 s and sonicated for 10 min. Another 80 μL of the IS working solution was added and vortexed for 10 s and sonicated for 10 min. Samples (200 μL) were then centrifuged for 10 min at $3000 \times g$ on a bench top centrifuge.

C18 solid phase extraction (SPE) cartridges (Varian Bond Elut 100 mg and 1 mL) were placed on a vacuum elution manifold and conditioned with 1 mL MeOH, followed by 1 mL water, taking care that the cartridges did not run dry at any stage. Only 170 μL of the prepared sample was applied to a cartridge and drawn through by applying low vacuum (±15 kPa). The loaded cartridges were washed with 1 mL water and then 1 mL MeOH was used to elute the adsorbed analytes. The cartridge bed was suctioned dry for 5 min. The eluent was gently evaporated under a gentle stream of nitrogen in a heater block at 48 °C. The dried residue was reconstituted with 50 μL of a mixture of 10% Solvent B and 90% Solvent A. Aliquots of 5 μL were injected onto the LC–MS/MS system.

2.5. Analytical method validation

The method was validated to meet the general requirements of ISO 17025 (2005) and SANS 17025 [12,13] and are discussed below.

2.5.1. Linearity

Daily standard calibration curves were constructed for EFV using the ratio of the observed analyte response and the response of the IS. The calibration curves were obtained by weighted $(1/x)$ linear regression. The calibration was established over the range of 3.125–100 μg/L.

2.5.2. Precision and accuracy

Each level ($n = 6$) of the calibration curve was measured daily before sample analysis. Throughout patient sample analysis, quality control samples were assayed. Quality control samples (6.25; 12.5; 25 and 50 μg/L respectively) were used for the precision and accuracy determination, the precision being calculated as the relative standard deviation (% RSD) of control samples within a single set of analyses run sequentially (intra-assay) and between different sets of analyses run on different days (inter-assays). The accuracy was determined by the Agilent MassHunter Software, Quantitative Application.

2.5.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was defined as the concentration where the analyte signal was at least 3 times greater than the background noise, while the LOQ was defined as the minimum concentration were the signal was 10 times greater than the background noise or the minimum
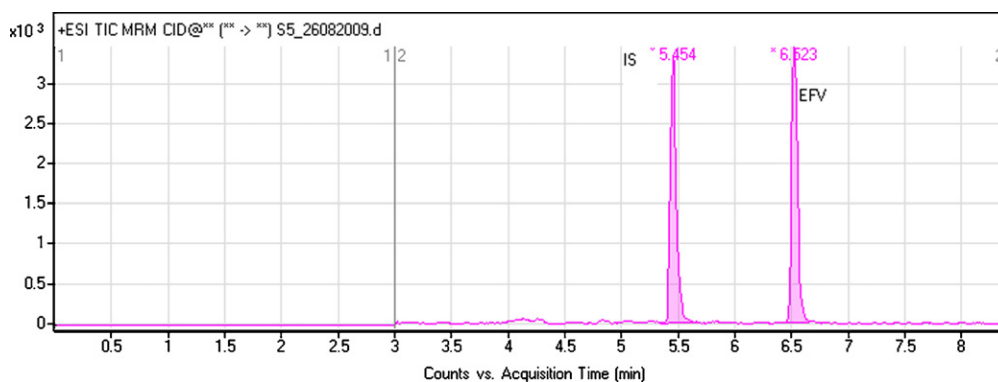
Fig. 1. Chromatogram of a saliva calibrant (EFV, 50 µg/L) with reserpine as the internal standard (IS).

concentration where the variance of determination was less than 15%.

2.5.4. Stability of EFV and the IS

Stability was determined by injecting the six separate saliva calibrators in triplicate (3.125, 6.25; 12.5; 25; 50; and 100 µg/L respectively) directly after sample preparation, then reinjecting the same calibrators again at 24 h and 48 h after preparation, while constantly keeping the calibrators at 4 °C in the auto-sampler tray.

2.5.5. Recovery

Three spiked MeOH/water (1:1) and equivalent saliva standards (12.5; 25 and 50 µg/L) were prepared from the same working solutions of EFV and IS. The saliva samples were processed according to the sample preparation protocol using SPE and each sample analysed by LC–MS/MS in triplicate. The MeOH/water standards were injected in triplicate without any further workup. The abundance for EFV of the saliva standards was evaluated against the abundance of the theoretical concentrations of unextracted MeOH/water (1:1). This assay was repeated 6 times.

2.5.6. Specificity

The specificity was confirmed using the developed method to analyse standards of the most common drugs employed in the treatment of HIV/AIDS and prophylaxis of opportunistic infections in the primary health care sector of South Africa using this method. Amoxicillin, acetaminophen, sulphametoxazole, trimethoprim, stavudine and lamivudine were dissolved in the appropriate solvents and injected, and by injecting double blank (containing no IS or EFV) and blank (containing only the IS) saliva samples from different donors who were not on any medication, after following the SPE protocol.

3. Results

3.1. Chromatograms

The proposed LC–MS/MS method enables the measurement of EFV in saliva in positive electrospray ionization mode. The retention times for the IS and EFV were 5.4 and 6.5 min respectively. Typical chromatographic profiles of a calibration sample (50 µg/L), a patient sample (6.90 µg/L) at 16.53 h post-last EFV dose, blank and double blank samples are shown in Figs. 1–4.

3.2. Analytical method validation

3.2.1. Linearity

The linear regression coefficient (r^2) of the calibration curves over a concentration range of 3.125–100 µg/L were between 0.989 and 0.992. The regression line equation was: $y = 0.1659x - 0.0546$.

3.2.2. Precision and accuracy

Precision and accuracy of samples at a low, medium and high concentration (6.25; 12.5; 25 and 50 µg/L respectively) are provided in Table 2.

Throughout these concentration ranges, the mean intra-assay precision was below 4%, and below 10% for the inter-assay precision. The accuracy for all three concentration levels was between 87% and 101%.

3.2.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was determined to be 1.84 µg/L and the LOQ was 6.11 µg/L with an assay variance of less than 15%. The working concentration range was therefore defined as 6.25–100 µg/L.
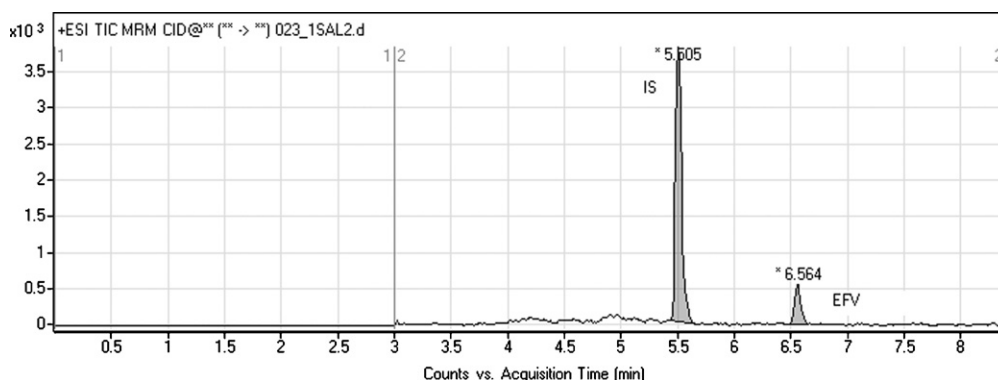

Fig. 2. Typical chromatogram of saliva from a patient on EFV (6.90 µg/L) antiretroviral therapy, 16.53 h post-last EFV dose, with reserpine as the internal standard (IS).
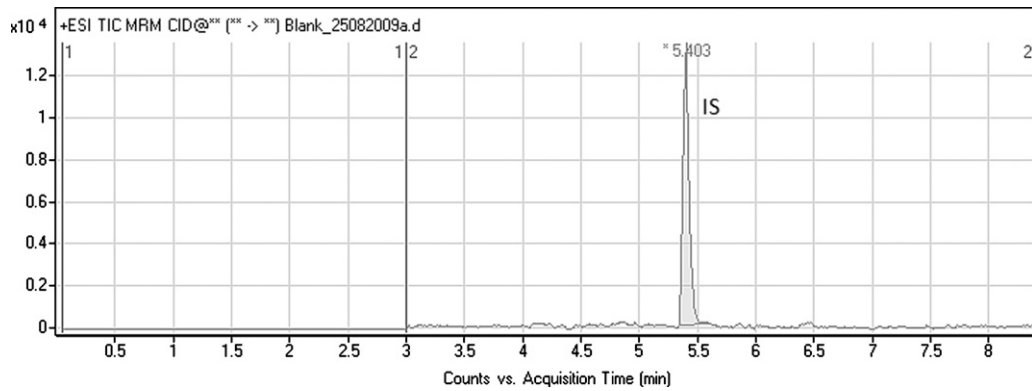
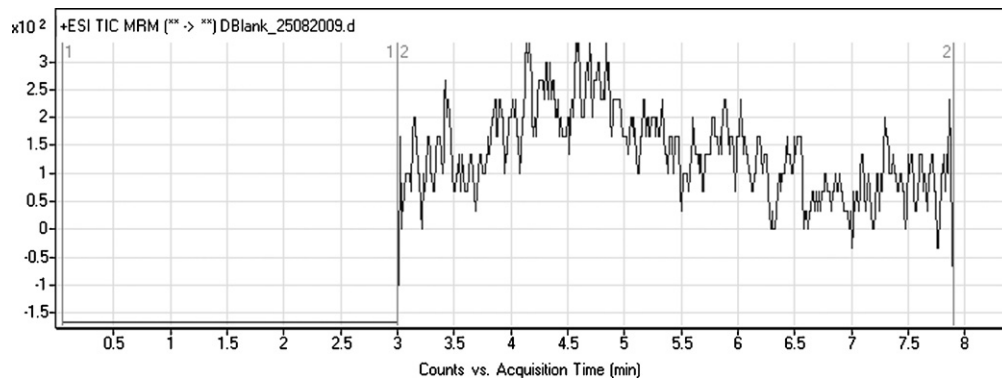**Fig. 3.** Chromatogram of a blank drug free saliva sample (containing IS).

Fig. 4. Chromatogram of a double blank saliva sample (containing no EFV or IS).

3.2.4. Stability

The stability of the samples left at 4 °C in the auto-sampler tray was determined at 24 h and 48 h after the initial injections which were performed directly after the calibrators were prepared. The average decrease for all six EFV concentrations measured (3.125; 6.25; 12.5; 25; 50 and 100 μg/L respectively) was 14% within the first 24 h which was still acceptable below 15% [12]. However, by 48 h the percentage decrease for the EFV for the same samples averaged 42%. The percentage decrease in the samples with the highest concentrations did show a smaller percentage change but still exceeded 15%, indicating that the samples were only stable for 24 h after sample workup if maintained at 4 °C.

3.2.5. Recovery

The mean absolute recovery for EFV measured with the high, medium and low controls were constant with a mean recovery of 60.66 ± 7.95%.

Table 2
Precision and accuracy of EFV in saliva.

Nominal concentration (μg/L)	Measured concentration (μg/L)	Precision (% RSD)	Accuracy (%)
Intra-assay (*n* = 3)[a]			
6.25	6.07	3.32	96.74
12.5	11.10	0.46	88.82
25	21.93	3.9	87.73
50	50.32	1.25	100.64
Inter-assay (*n* = 5)			
6.25	6.07	6.10	98.40
12.5	11.13	8.29	89.04
25	22.00	4.60	87.93
50	50.35	9.43	100.70

[a] Only 3 samples could be assayed due to the low volume of sample in the vial.

3.2.6. Specificity

The chromatograms for amoxicillin, acetaminophen, sulphametoxazole, trimethoprim, lamivudine, stavudine and the blanks showed no interfering peaks at any retention time during the separation. The method specificity was confirmed by analysing all these drugs with the same method and it showed that the method was specific for EFV and reserpine.

4. Discussion and conclusion

This validated LC–MS/MS method provides a novel, robust screening procedure for determining salivary EFV obtained from HIV-1-infected individuals which is sensitive enough to confirm compliance in taking the antiretroviral therapy. The method has a high specificity and requires a relatively small sample volume that is easily obtainable and non-invasive.

Several analytical methods have been published for the analysis of EFV in plasma, either alone or in combination with other drugs, using HPLC–UV [14–17] and LC–MS/MS [18–23] techniques. Determination of EFV levels in human hair [7] and reports of determining nevirapine [8,10] and indinavir [24] from saliva have been published, but this study is the first validated LC–MS/MS method for the assay of EFV in saliva to be reported.

The method reported here incorporates positive electrospray ionization compared to the use of negative electrospray ionization reported by most published articles [19,23,25], allowing for the possibility to detect other antiretroviral drugs in a single run.

One challenge of this assay was the sample preparation method that uses offline solid phase extraction with an evaporation step that is time consuming, but this could be overcome using an on-line SPE technique.

The recovery is low but consistent. This could be due to the use of the Salivette® for the saliva collection which does tend to have

a poor recovery for lipophilic drugs [26]. The limits of detection and quantification show that the method is sensitive and specific enough to detect the low concentrations of EFV in saliva of patients on ARV treatment. This has the advantage of using a non-invasive sample collection. A challenge that was experienced with the saliva collection was that only a small volume of saliva could be collected, probably due to the subjects chewing too hard on the cotton wool. Therefore, only a small sample volume (100 μL) could be used for the analysis. A larger sample volume could possibly increase the sensitivity of this method. We would suggest that the correlation between unbound EFV plasma concentrations and the EFV saliva concentrations be further explored to investigate the possibility of saliva analysis as alternative for EFV TDM or as a screening tool for EFV compliance.

Disclosure statement

Acknowledgements

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