Compartmentalization and Antiviral Effect of Efavirenz Metabolites in Blood Plasma, Seminal Plasma and Cerebrospinal Fluid

Lindsay B. Avery, Jennifer L. VanAusdall, Craig W. Hendrix, Namandjé N. Bumpus

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287, USA (LBA, JLV, CWH, NNB)

Department of Medicine, Division of Clinical Pharmacology, The Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287, USA (CWH)
DMD #49601

Running Title:
Compartmentalization and Antiviral Effect of EFV Metabolites

**Corresponding Author:** Namandjé N. Bumpus, Ph.D., Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Biophysics 307-A, Baltimore, MD 21205, USA. Phone: (410)-955-0562. Fax: (410)-955-3023.

Email address: nbumpus1@jhmi.edu

---

**Number of Text Pages:** 28

**Number of Tables:** 0

**Number of Figures:** 6

**Number of References:** 42

**Number of Words in Abstract:** 250

**Number of Words in Introduction:** 617

**Number of Words in Discussion:** 1361

**Abbreviations:**

EFV, Efavirenz; 8-OH EFV, 8-hydroxy efavirenz; 7-OH EFV, 7-hydroxy efavirenz; 8,14-OH EFV, 8,14-dihydroxy efavirenz; ARV, antiretroviral drug; CYP, a cytochrome P450; CNS, central nervous system; HLM, human liver microsome; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; logP, lipophilicity partitioning
Abstract

Efavirenz (EFV) is one of the most commonly prescribed antiretrovirals for use in the treatment of HIV infection. EFV is extensively metabolized by the cytochromes P450 (CYP) to a number of oxygenated products; however, the pharmacologic activity and distribution of these metabolites in anatomical compartments have yet to be explored. The systemic distribution of EFV oxidative metabolites was examined in blood plasma, seminal plasma, and cerebrospinal fluid from subjects on an EFV-based regimen. The 8-hydroxy EFV metabolite was detected in blood plasma, seminal plasma and cerebrospinal fluid, with median concentrations of 314.5ng/ml, 358.5ng/ml, and 3.37ng/ml, respectively. In contrast, 7-hydroxy and 8,14-hydroxy EFV were only detected in blood plasma and seminal plasma with median concentrations of 8.84ng/ml and 10.23ng/ml, and 5.63ng/ml and 5.43ng/ml, respectively. Interestingly, protein-free concentrations of metabolites were only detectable in seminal plasma, where a novel dihydroxylated metabolite of EFV was also detected. This accumulation of protein-free EFV metabolites was demonstrated to be the result of differential protein binding in seminal plasma compared to that of blood plasma. In addition, the oxidative metabolites of EFV did not present with any significant pharmacological activity towards HIV-1 as measured using an HIV-GFP single round infectivity assay. This study is the first to report the physiological distribution of metabolites of an antiretroviral into biological compartments which the virus is known to distribute, and to examine their anti-HIV activity. These data suggest that the male genital tract may be a novel compartment that should be considered in the evaluation of drug metabolite exposure.
Introduction

HIV infection distributes to a variety of anatomic sites including but not limited to the circulatory system, central nervous system (CNS), genital tracts, lymphatic system and intracellular compartments. Antiretroviral drugs (ARVs) have been highly effective in the treatment of HIV through suppression of viral replication; however, there is a lack of understanding in ARV distribution beyond the blood plasma thereby limiting our ability to evaluate extravascular efficacy and/or toxicity. A major anatomical site of concern in the treatment of HIV is the CNS since the virus is known to distribute to this compartment yet many ARVs exhibit poor penetration into the cerebrospinal fluid (Letendre S and et al., 2008; Dellamonica et al., 2012). HIV associated neurological disease is relatively common, resulting in significant cognitive and neurological impairment, however the etiology is incompletely understood (Resnick et al., 1988; Sacktor, 2002; McArthur, 2004; Valcour et al., 2012). The male genital tract has also shown poor ARV penetration (Kashuba et al., 1999; Avery et al., 2011) and is a compartment of concern in that it is the primary site of transmission through HIV-laden semen (Resnick et al., 1988; Stekler et al., 2008; HIV/AIDS, 2009). Although the ability of ARVs to penetrate these compartments is an important aspect of HIV therapy, in many cases, a comprehensive understanding of the distribution of these drugs beyond the blood is lacking. One of the better characterized drugs in this regard is the non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) that has been demonstrated to penetrate, though minimally, both the male genital tract and CNS. Recent reports have revealed that there exists a 150-fold total concentration gradient for EFV from blood plasma to cerebrospinal fluid (Best et al., 2011) and 20-fold from blood plasma to seminal plasma (Avery et al., 2011).

EFV is extensively metabolized by the cytochrome P450 (CYP) superfamily of heme containing mono-oxygenases to yield several products including 8-hydroxyEFV (8-OH EFV),...
which is the major oxygenated metabolite of efavirenz and is formed by CYP2B6 (Ward et al., 2003; Bumpus et al., 2006). EFV is also metabolized to a lesser extent to 7-hydroxy efavirenz (7-OH EFV) and 8,14-hydroxy efavirenz (8,14-OH EFV) that are formed by CYP2A6 and CYP2B6, respectively (Mutlib et al., 1999; Bumpus et al., 2006; Ogburn et al., 2010). In addition, EFV is an autoinducer of metabolism in that it increases expression of CYP2B6 through activation of the constitutive androstane receptor (Faucette et al., 2007). These enzymes have been shown to be detectable in both the blood brain barrier and the prostate suggesting the possibility for local metabolism of EFV in the central nervous system and male genital tract (Finnström et al., 2001; Miksys and Tyndale, 2002; Kumagai et al., 2007). Thus, the studies herein were designed to gain an understanding of the distribution of metabolites of EFV in the blood plasma, seminal plasma and cerebrospinal fluid while also examining their anti-HIV activity. As such, the following novel findings are reported: 1) mono- and di-oxygenated metabolites of EFV are present at similar levels in seminal plasma and blood plasma; 2) protein-free EFV metabolite concentrations were only present in seminal plasma and presented with different degrees of protein binding compared to blood plasma; 3) mono- and di-oxygenated EFV metabolites are not pharmacologically active towards HIV; 4) a previously unreported dihydroxylated metabolite of EFV that appears to be seminal plasma specific was detected. Taken together, this study is the first, to the best of our knowledge, to report the physiological distribution of metabolites of an ARV into relevant biological compartments and to examine their anti-HIV activity. Further, these data suggest that the male genital tract may be a novel compartment that should be considered in working to gain a comprehensive understanding of drug metabolite exposure.
Materials and Methods

Subjects and demographic characteristics

Archived blood plasma and cerebrospinal fluid samples were received from a previous study (Tovar-y-Romo et al., 2012), and analyzed for total and protein-free EFV and EFV metabolite concentrations. The study involved 13 total participants on a once daily dosing regimen containing EFV, where paired blood plasma and cerebrospinal fluid samples were obtained. All subjects had been taking EFV for a minimum of 4 weeks prior to study enrollment. The 13 total research participants ranged in age from 37 to 71 years old, of which 2 were women and 11 were men, and 1 was European American and 12 were African American.

Archived blood plasma and seminal plasma samples were obtained from an additional previous study (Cao et al., 2007) and analyzed for total and protein-free EFV and EFV metabolite concentrations. The original study involved 6 total research participants who provided paired blood and semen samples at multiple time points throughout a 5 day study. Research participants on 600mg daily EFV were changed to a 100mg EFV every 4 hour regimen to establish near true steady-state conditions due to the estimated 40-55 hour EFV half-life and frequent dosing interval. The six men enrolled ranged in age from 33 to 48 years old, of which 1 was European American and 5 were African American.

Each of these studies was approved by the Johns Hopkins Medicine Institutional Review Board and all research participants provided informed consent for participation.

Materials

Efavirenz was obtained through the NIH Aids Research and Reagent Program (Germantown, MD). Synthetic 8-OH EFV, 7-OH EFV and 8,14-OH EFV were obtained from Toronto Research
Chemicals (Toronto, Ontario, Canada). A racemic 6-fluorinated analog of EFV (F-EFV) for use as an internal standard was synthesized by Dr. David Meyers (Johns Hopkins University School of Medicine Synthetic Core Facility located in the Department of Pharmacology and Molecular Sciences) using modifications of previously published methods (Radesca et al., 1997; U.S.FDA, 2001). EFV, 8-OH EFV, 7-OH EFV and 8,14-OH EFV were dissolved in DMSO for infectivity assays and in acetonitrile for metabolism assays and stored at -20°C. Concentrations of solvent vehicles were less than 0.1% in all experiments.

**Separation of Protein-free from Protein-Bound by Ultrafiltration**

Separation of protein-free from protein bound was performed according to a previously described method (Avery et al., 2011). Briefly, ultrafiltration of samples was performed using 96 well plates with a 10kDa filter membrane (Millipore, Billerica, MA, USA). Blank blood plasma for quality control preparation and method development was obtained from Biological Specialty Corporation (Colmar, PA, USA). Blank seminal plasma was obtained from Bioreclamation, Inc. (Westbury, NY, USA). To each well of the ultrafiltration plate, 100 µl of sample was added. Samples were incubated for one hour at 37°C. The plates were centrifuged at 15 minute intervals for 45 minutes at 37 °C. At every 15 minute interval, the filtrate was collected in a v-bottom 96 well collection plate and extracted with methanol. Filtrate samples were then analyzed for EFV and EFV metabolite concentrations by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The free EFV concentration in the clinical samples evaluated at the three centrifugation time points were used to generate an EFV concentration vs. centrifugation time curve for each clinical sample. The centrifugation time-adjusted free EFV concentration was determined by using a linear regression to estimate the concentration when
time equals zero minutes (unperturbed pre-centrifugation sample). Percent protein binding was calculated as the centrifugation time-adjusted free drug concentration divided by the total drug concentration multiplied by 100.

**Infectivity Assay**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using ficoll-paque PLUS (GE Healthcare Biosciences; Piscataway, NJ), according to the manufacturer’s instructions. Isolated cells were resuspended in a stimulation medium of RPMI 1640 supplemented with 10% FBS (Invitrogen, Grand Island, NY), 100U/mL interleukin-2 (IL-2, Proleukin), Penicillin/Streptomycin, and activated with 0.5µg/mL phytohemagglutinin (PHA) (Fisher Scientific; Pittsburgh, PA). Cell suspensions were incubated for 3 days at 37°C and 5% CO2. CD4+ T cells were isolated using magnetic activated cell sorting (MACS) CD4+ T cell isolation kit (Miltenyi Biotec; Cambridge, MA) according to the manufacturer’s instructions.

Inhibition of HIV-1 infection was measured using a modification of a previously established method using an HIV-1 reporter virus expressing an Env-green fluorescent fusion protein (GFP) (Zhang et al., 2004; Shen et al., 2008; Sampah et al., 2011; Jilek et al., 2012). Infections were performed in an assay medium containing RPMI 1640 + 50% FBS, 100U/mL IL-2, and Penicillin/Streptomycin. CD4+ T cells were seeded at 1 x 10^5 cells per well and incubated with EFV, 8-OH EFV, 7-OH EFV or 8,14-OH EFV for 3 hours prior to infection. DMSO solvent vehicle was less than 0.1% for all experiments. Infection was performed via spinoculation (O'Doherty et al., 2000), incubated for 72 hours, and analyzed for infection by flow cytometry analysis of GFP expression. Infectivity for each sample (\( f_a(SAMPLE_X) \)) is characterized as the fraction of virus infection events affected by the drug (\( f_a \)) relative to the
fraction of virus infection events unaffected by drug ($f_u$). Infectivity is determined relative to the positive control (HIV-GFP virus only, no drug) and negative control (no virus, no drug) for each sample set. The data from infectivity experiments is calculated as follows:

Equation 1:

$$\text{Relative Infectivity} (\%) = \frac{f_u (\text{SAMPLE X})}{f_u (\text{pos.control}) - f_u (\text{neg.control})} \times 100$$

where $f_u = 1 - f_a$.

Metabolism Assays

To analyze metabolite formation, EFV, 8-OH EFV, and 7-OH EFV were incubated at 5 µM or 20 µM with human liver microsomes (HLM) (50 donor pool; BD Biosciences, San Jose, CA) as well as cDNA expressed individual human cytochromes P450 (BD Biosciences; CYP1A1, -1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -3A4, -3A5, and -3A7). The final concentration of cDNA expressed P450s and HLM were 20 pmol/mL and 1mg/mL, respectively. The final reaction volumes for assays performed using cDNA expressed P450s and HLM were 100 µL and 500 µL, respectively. Following a 5 minute equilibration period in 100mM potassium phosphate buffer (pH 7.4) at 37°C, the reaction was initiated by addition of an NADPH regenerating system (BD Biosciences). The reaction was incubated for 60 minutes at 37°C. All reactions were terminated by the addition of acetonitrile. Metabolite products were extracted using a liquid: liquid extraction method, where 600 µl of 50mM Ammonium Formate was added to the reaction mixture, followed by 900 µl of Hexane:Ethyl Acetate (1:1). The reaction mixture was vortexed, centrifuged at 3000xg for 10 minutes at 4°C, and the organic layer was aliquoted into a separate tube. The organic layer was evaporated to dryness under nitrogen gas, the residue was reconstituted in 200 µl of methanol and 5µl was injected for UPLC-MS/MS analysis.
UPLC-MS/MS Detection of EFV and Metabolites

EFV and EFV metabolites were detected and quantified using a UPLC-MS/MS assay performed on an AB Sciex QTRAP® 5500 (AB Sciex, Foster City, CA) interfaced with an Acquity® UPLC (Waters Inc., Milford, MA). EFV and EFV metabolite samples were analyzed according to a modification of a previously established method (Avery et al., 2010). EFV and EFV metabolites were resolved using a reverse-phase UPLC column (2.1 × 50 mm Acquity UPLC BEH C18) with a flow rate of 0.5 mL/min by gradient elution [mobile phase A (MPA) of 0.1% formic acid in water; mobile phase B (MPB) of 0.1% formic acid in acetonitrile]. At initial conditions, samples are injected at solvent conditions of 70% MPA and 30% MPB. From 0.4 minutes to 4.5 minutes MPB is increased to 85% and then 100% MPA at 4.6 minutes to elute EFV and EFV metabolites. Column conditions are equilibrated to 85% MPA and 15% MPB from 4.8 minutes to 5.5 minutes. EFV and EFV metabolites were detected via negative-ion multiple reaction monitoring (MRM). The assay was linear from 0.5 – 500 ng/mL with assay characteristics consistent with the FDA Guidance for Industry (U.S.FDA, 2001) where all calibration standards and quality controls were linear with percent deviation and coefficient of variation of less than or equal to 15%. A fluorinated analog of EFV (F-EFV) was employed as the internal standard as previously described (Avery et al., 2010). EFV, 8-OH EFV, 7-OH EFV, 8,14-OH EFV and F-EFV were detected via the MRM transitions: m/z 314.0 > 244.1, m/z 329.9 > 162.0, m/z 329.9 > 188.9, m/z 346.0 > 262.0, and m/z 298.0 > 227.9, respectively.
Results

Quantitation of EFV Metabolite Concentrations in Blood Plasma, Seminal Plasma and Cerebrospinal Fluid.

Total concentrations of EFV were previously established in the paired blood plasma and seminal plasma samples with median (interquartile range (IQR)) concentrations of 2360 ng/ml (1530 – 4120) and 95 ng/ml (70 – 206), respectively (Avery et al., 2011). Total concentrations of EFV were previously measured in the paired blood plasma and cerebrospinal fluid samples with median (IQR) concentrations of 2170 ng/ml (1896 – 2520) and 19 ng/ml (7 – 24) (Tovar-y-Romo et al., 2012). In order to measure the concentrations of 8-OH EFV, 7-OH EFV, and 8,14-OH EFV in blood plasma, seminal plasma and cerebrospinal fluid, we developed a novel UPLC-MS/MS assay. Using this assay it was determined that 8-OH EFV, the predominant metabolite of EFV, was detectable in blood plasma, seminal plasma and cerebrospinal fluid with median (interquartile range (IQR)) concentrations of 314.5 ng/ml (206 – 362.3), 358.5 ng/ml (340 – 368.8), and 3.37 ng/ml (2.58 – 6.54), respectively. In contrast, 7-OH EFV was only detected in blood plasma and seminal plasma, with median (IQR) concentrations of 8.84 ng/ml (6.21 – 12.48) and 10.23 ng/ml (8.26 – 12.23), respectively. The 8,14-OH EFV metabolite was quantifiable in blood plasma and seminal plasma with median (IQR) concentrations of 5.63 ng/ml (4.58 – 6.16) and 5.43 ng/ml (4.15 – 6.10). Only two out of thirteen subjects had detectable 8,14-OH EFV in the cerebrospinal fluid with concentrations of 0.375 and 0.444 ng/ml, respectively (Figure 1). Qualitatively, it was also found that N- and O-linked glucuronidated metabolites of EFV were present in both blood plasma and seminal plasma and that the abundance of these glucuronide conjugates was similar in the two compartments (data not shown).
In order to measure the protein-free concentrations of the EFV metabolites an established ultrafiltration method (Avery et al., 2011) was used. Protein-free concentrations of EFV metabolites were only detectable in seminal plasma with median (IQR) concentrations of 10.16 ng/ml (7.29 – 42.36) for 8-OH EFV, 6.55 ng/ml (5.14 – 22.18) for 7-OH EFV and 4.04 ng/ml (3.98 – 5.80) for 8,14-OH EFV (Figure 1). As an explanation for the differences observed in both the total concentration gradients and the detection of protein-free metabolites only in seminal plasma, protein binding was examined for each metabolite. Since protein-free concentrations were not detectable at concentrations greater than the lower limit of quantitation (0.5 ng/mL) in blood plasma from clinical samples, concentrations of EFV metabolites were added to blank lots of blood plasma to provide a comparison to seminal plasma. The median protein binding of EFV, 8-OH EFV, 7-OH EFV, and 8,14-OH EFV in blood plasma was 99.82%, 99.97%, 99.62%, and 99.61%, respectively. The median protein binding of EFV, 8-OH EFV, 7-OH EFV, and 8,14-OH EFV in seminal plasma was 95.10%, 96.20%, 38.09%, and 15.42%, respectively.

Formation of Dihydroxylated Metabolites of EFV

Interestingly, analysis of the metabolite profiles in each anatomic compartment revealed that in addition to 8,14-OH EFV (Figure 2, retention time 1.56 min) a second dihydroxylated metabolite (Figure 2, 1.73 min) was detectable above background in seminal plasma. Previous studies examining the oxidative metabolism of EFV had identified 8,14-OH EFV as the sole dihydroxylated metabolite of EFV in humans (Mutlib et al., 1999; Ward et al., 2003). A recent study has identified a second dihydroxylated metabolite of EFV that was observed in vivo and in vitro following incubation with either 7- or 8-OH EFV as a substrate (Ogburn et al., 2010). With this in mind, in order to investigate the formation of this potentially novel metabolite in vitro...
studies were performed using human liver microsomes with EFV, 7-OH EFV or 8-OH EFV as the substrate. As shown in Figure 3, the 1.56 and 1.73 min dihydroxylated metabolites were only detectable in the reactions containing 8-OH EFV as the substrate, suggesting that formation of these metabolites involved oxygen insertion at the 8-position of EFV. By Contrast, a third metabolite was detectable at 1.92 min following incubation with EFV, 7-OH EFV, and 8-OH EFV, suggesting that formation of this metabolite may involve oxygen insertion at both the 7-position and 8-position of EFV.

In order to probe the structures of these three dihydroxylated products tandem mass spectrometry (MS/MS) was performed (Figure 4). The fragmentation pattern of the 1.56 min retention time metabolite is commensurate with that of 8,14-OH EFV as previously described (Mutlib et al., 1999). We propose that the m/z 282.0, 262.0, 232.0, and 167.7 fragment ions of the 1.73 min retention time metabolites result from the loss of CH2O2 and H2O, C4H4O2, C5H6O3, and C7H11OF3, respectively. The daughter ions 302.0, 273.9, 262.0, and 210 of the 1.92 min retention time metabolite are proposed to correspond to the loss of CO2, C3H4O2, C4H4O2, and C6H7F3, respectively. The three observed dihydroxylated products exhibit highly similar fragmentation patterns; however, the fragment ion of m/z 210.0 for the 1.92 min retention time metabolite may indicate dihydroxylation on the aromatic ring as opposed to both the aromatic ring and the cyclopropyl ring. Thus, this metabolite may be a 7,8-OH EFV product as previously hypothesized (Ogburn et al., 2010). In contrast, the m/z 168.0 fragment of the 1.73 min retention time metabolite suggests hydroxylation on the cyclopropyl ring similar to 8,14-OH EFV. Such a metabolite has yet to be reported. With this in mind, we employed cDNA-expressed P450s in order to identify the enzyme(s) involved in the formation of this product. The panel of CYPs
tested included CYPs 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5 and 3A7. The
dihydroxylated metabolites retained at 1.56 and 1.73 minutes were solely formed using 8-OH
EFV as a substrate and the formation of these products was primarily catalyzed by CYP2B6, as
well as CYP1A1 to a lesser extent. The dihydroxylated metabolite retained at 1.92 minutes was
formed primarily by CYP2B6 with 8-OH EFV as a substrate. In addition, this product was also
detected in incubations containing CYP2B6 and CYP2C8 with substrates EFV and 7-OH EFV,
respectively (Figure 5). CYP2B6 has been previously demonstrated to be responsible for
metabolizing EFV to 8-OH EFV and 8,14-OH EFV (Ward et al., 2003). To summarize the
formation of dihydroxylated metabolites, we propose a schematic in Figure 6, consistent with
previously determined oxidative metabolism of EFV (Mutlib et al., 1999; Ward et al., 2003;
Ogburn et al., 2010).

**HIV-Inhibitory activity of EFV Metabolites**

Since we were able to detect metabolites of EFV in extravascular compartments where HIV can
distribute, we used a single round infectivity assay (Zhang et al., 2004; Shen et al., 2008;
Sampah et al., 2011; Jilek et al., 2012) to investigate the anti-HIV activity of synthetic 8-OH
EFV, 7-OH EFV and 8,14-OH EFV. Data demonstrating whether or not metabolites of EFV
contribute to the inhibition of HIV activity of EFV have yet to be reported. Compared to the
experimentally observed IC$_{50}$ of 0.36 ng/ml for EFV – 8-OH EFV, 7-OH EFV, and 8,14-OH
EFV had IC$_{50}$ values of 42.25 ng/ml, 44.68 ng/ml, and 2238.4 ng/ml, respectively. Our
experimentally determined IC$_{50}$ value for EFV is comparable to previously established wild-type
*in vitro* IC$_{50}$ for EFV of 0.51 ng/mL (Parkin et al., 2004).
Discussion

EFV is one of the most commonly used ARVs in the treatment of HIV. Although EFV is extensively metabolized there is a lack of understanding of the distribution and pharmacological effect of the metabolites of EFV. This study is the first to demonstrate physiological distribution of metabolites of an ARV into relevant biological compartments and examine the role which metabolism may play in antiviral activity. Concentrations of 8-OH EFV, 7-OH EFV, and 8,14-OH EFV were detectable in biological matrices of blood plasma, seminal plasma, and cerebrospinal fluid of patients on an antiretroviral regimen containing EFV.

Interestingly, EFV has been previously demonstrated to induce compartment specific toxicities including hepatotoxicity, CNS toxicity and decreased semen quality (Sulkowski et al., 2002; Luz et al., 2003; Dieterich et al., 2004; Ciccarelli et al., 2011). Recently, we demonstrated for the first time that 8-OH EFV has the ability to induce cytotoxicity via stimulation of mitochondrial dysfunction and activation of stress activated signaling pathways (Bumpus, 2011). Subsequently, toxicity of 8-OH EFV to dendritic cells of the CNS has also been reported (Tovar-y-Romo et al., 2012). Although the mechanisms underlying adverse effects of EFV on semen quality have yet to be fully elucidated it has been reported that treatment with EFV resulted in decreased motile spermatozoa and vitality, suggested to be the result of mitochondrial toxicity or direct toxicity to the cells producing spermatozoa (van Leeuwen et al., 2008; Lambert-Niclot et al., 2011). Since 8-OH EFV has been reported to play a causal role in certain compartment specific toxicities (Bumpus, 2011; Tovar-y-Romo et al., 2012), understanding the clinical exposure of mono- and di-hydroxylated metabolites of EFV is of importance.

We have reported previously that protein-free concentrations of EFV are in equilibrium in the blood plasma and seminal plasma resulting from different degrees of protein binding in
each compartment which create the very large total drug concentration gradients (Avery et al., 2011). This suggests that EFV distributes into the male genital tract by mechanisms of passive diffusion. By contrast, for the mono- and di-hydroxylated metabolites of EFV, we have demonstrated in this study that the protein-free metabolites do not exist in equilibrium in the two compartments, but rather are found at measurable concentrations only in the seminal plasma, and are below assay detection limits, if at all, in blood plasma and cerebrospinal fluid. Although the protein-free concentrations of EFV metabolites were not detectable in the blood plasma samples, the protein-free gradients may be estimated using the \textit{in vitro} determinations of metabolite protein binding in blood plasma and median total concentration determined for each metabolite. Protein-free 8-OH EFV, 7-OH EFFV and 8,14-OH EFV may therefore have seminal plasma: blood plasma free drug concentration gradients as high as 145, 188, and 209-fold, respectively.

One possible explanation for these gradients would involve EFV metabolites being potential substrates for human drug transporters at the blood-testis barrier, causing an influx of these molecules within the compartment. In the event of local metabolism of EFV, this could result in reduced compartment-specific concentrations of parent compound resulting in reduced pharmacologic effect, or an increased concentration of metabolites, which, may be pharmacologically active with a desirable effect or may induce local toxicity.

While the protein binding of 8-OH EFV is similar to EFV in both blood plasma and seminal plasma, the protein binding of 7-OH EFV and 8,14-OH EFV was found to be significantly reduced compared to the parent compound. These differences suggest that chemical modification by hydroxylation impacts the affinity with which the compounds bind to plasma proteins, which may impact the overall distribution of each compound. If mono- and di-hydroxylated metabolites of EFV are primarily transported by passive diffusion, accumulation
may be the result of protein binding, lipophilicity partitioning (logP), and/or ion trapping (Kashuba et al., 1999). Seminal plasma, composed primarily of secretions from the prostate glands and seminal vesicles, is the main fluid assessed when analyzing the male genital tract (Klemmt and Scialli, 2005). Weak acids have previously been demonstrated to accumulate in prostatic fluid by ion-trapping mechanisms (Winningham et al., 1968). Mono- or dioxygenation of a compound can impact both the acid dissociation constant and lipophilicity (partition coefficient, logP) of a molecule which can have a marked impact on compound disposition (Mack and Bönisch, 1979; Kem et al., 2004). In oxidative metabolism, the log P is typically shown to reduce for a compound as it is transformed to be more hydrophilic (Manners et al., 1988), which also impacts the acidity and basicity (pKa) of a compound. For EFV metabolites, it is our hypothesis that the observed seminal plasma accumulation is the result of these fundamental chemical changes to EFV resulting from primary oxidative metabolism.

To date, it has yet to be demonstrated if the metabolites of EFV contribute to the anti-HIV activity of EFV. Using an HIV pseudovirus with an env-GFP fusion protein, we have analyzed the impact of 8-OH EFV, 7-OH EFV, and 8,14-OH EFV on viral infectivity. Based on the clinical concentrations observed for 8-OH EFV and 7-OH EFV, these results suggest 8-OH EFV and 7-OH EFV are minimally effective at inhibiting viral replication. While clinically observed concentrations were greater than our experimentally determined IC50 particularly for 8-OH EFV, because of the high degree of protein binding there is insufficient protein-free drug available in biological matrices to contribute to the pharmacological activity of EFV.

Using our highly sensitive UPLC-MS/MS method, we have demonstrated the production of three distinct dihydroxylated metabolites, with proposed fragmentation patterns and mechanism of formation based on *in vitro* and *in vivo* characterization. The metabolism of EFV
to 8-OH EFV, 7-OH EFV, and 8,14-OH EFV was originally established by Mutlib et al., 1999, which identified 17 potential metabolites of EFV using HPLC and NMR. More recently, Ogburn et al. 2010, described an observed dihydroxylated metabolite from EFV after incubation with 7-OH EFV and 8-OH EFV, primarily formed by CYP 2B6. Consistent with their results, we propose this metabolite to be a 7,8-dihydroxylated metabolite. In addition, a third dihydroxylated metabolite of EFV was also detected, and shared fragmentation patterns similar to 8,14-OH EFV, indicating this metabolite may also be hydroxylated on the cyclopropyl ring of EFV. Because the HPLC methods used in prior analyses did not have the sensitivity and resolution of our methodology, it is likely they did not have the ability to distinguish or separate multiple dihydroxylated metabolites. Many of the fragmentation ions proposed by Mutlib et al. 1999, characteristic of 8,14-OH EFV are also characteristic of the two novel dihydroxylated metabolites of EFV proposed here and by Ogburn et al. 2010, suggesting that they were not able to differentiate between the multiple products. In addition, our results have also shown that the primary dihydroxylated metabolite of 8,14-OH EFV in vivo differs from the primary metabolite of 7,8-OH EFV in vitro using HLMs. This is consistent with Mutlib et al. 1999, as 8,14-OH EFV was originally isolated and characterized from in vivo samples, from the urine of guinea pigs.

In summary, we have established the distribution of the primary oxidative metabolites of EFV, 8-OH EFV, 7-OH EFV, and 8,14-OH EFV, and defined the role of protein binding in extravascular compartments. We have characterized the concentration profiles of EFV metabolites in three anatomic compartments relevant to HIV infection: the blood plasma of the circulatory system, the seminal plasma of the male genital tract, and the cerebrospinal fluid of the central nervous system. Interestingly, we have shown how there is significant accumulation into the seminal plasma of hydroxylated metabolites of EFV which may impact local
pharmacology and/or toxicology. Future work may involve examination of the potential for local
toxicity in compartments such as the male genital tract where we have found oxidative
metabolites to accumulate, including 8-OH EFV, which has been demonstrated to induce
compartment specific mechanisms of toxicity (Bumpus, 2011; Tovar-y-Romo et al., 2012).
Taken together, this study is the first to demonstrate the anatomic distribution of metabolites of
an ARV into relevant biological compartments and to examine their anti-HIV activity. These
results suggest there may be important compartmental differences in EFV metabolite
distribution, and the male genital tract may be a novel compartment in understanding local drug
distribution, efficacy, and toxicity of oxidative metabolites.

Author Contributions

Participated in Research Design: Avery, Bumpus

Conducted Experiments: Avery, VanAusdall

Contributed New Reagents or Analytical Tools: Hendrix

Performed Data Analysis: Avery, VanAusdall, Bumpus

Wrote or Contributed to the Writing of the Manuscript: Avery, VanAusdall, Hendrix, Bumpus
References:


Their Molecular Properties, Interactions with Brain Nicotinic Receptors, and Brain Penetration. *Molecular Pharmacology* **65:**56-67.


Footnotes

The following work was supported by a pharmacology/toxicology research starter grant from the Pharmaceutical Research and Manufacturers of America Foundation awarded to Dr. Namandje N. Bumpus. The AB SCIEX QTRAP® 5500 mass spectrometer was purchased using National Institutes of Health grant [1S10 RR 27733] and the Waters Acquity ultra-performance liquid chromatograph (UPLC) interfaced with the AB SCIEX QTRAP® 5500 was purchased with funds provided by the Pendleton Foundation Trust awarded to Dr. Craig W. Hendrix of the Johns Hopkins University School of Medicine.
Figure 1. Distribution of EFV and Hydroxylated EFV Metabolites into Blood Plasma, Seminal Plasma, and Cerebrospinal Fluid. Total concentrations of 8-OH EFV, 7-OH EFV, and 8,14-OH EFV were analyzed in subjects on a regular EFV-based regimen and found detectable in blood plasma (▼, panel A), seminal plasma (●, panel B), and cerebrospinal fluid (▲, panel C). Protein-free (○) concentrations of 8-OH EFV, 7-OH EFV, and 8,14-OH EFV were only detectable in SP (panel B).

Figure 2. Physiological Formation of Di-hydroxylated EFV Metabolites. The production of a second di-OH EFV metabolite (1.73 min retention time), distinct from 8,14-OH EFV (1.56 min retention time) was determined in clinical matrices from subjects on a steady state EFV-containing regimen. The panels depict the MRM transition of m/z 346>262 for (A) Blood plasma. (B) Seminal Plasma. (C) Cerebrospinal fluid from an individual subject.

Figure 3. Formation of Di-hydroxylated EFV Metabolites from Human Liver Microsomes. Production of dihydroxylated EFV metabolite following substrate incubation with human liver microsomes. The panels depict the common MRM transition of m/z 346>262 for human liver microsomes incubated with (A) 20µM EFV, (B) 20µM 8-OH EFV, and (C) 20µM 7-OH EFV.

Figure 4. Fragmentation Patterns of Dihydroxylated Metabolites of EFV. MS/MS fragmentation patterns were determined for each distinct dihydroxylated EFV metabolite with retention times of 1.57 min (panel A), 1.73 min (panel B), and 1.92 min (panel C).
Figure 5. Contribution of Individual CYPs to the Formation of EFV Metabolites. Individual cDNA-expressed CYPs were incubated with 5 μM EFV, 8-OH EFV, or 7-OH EFV and analyzed by UPLC-MS/MS for the formation of dihydroxylated products of EFV at 1.57 min (A) 1.74 min (B), and 1.92 min(C). The data are presented as the mean ± SD of n=3.

Figure 6. Proposed Schematic of EFV Mono- and Di- Oxidative metabolism. EFV metabolism to 8-hydroxy-EFV catalyzed by CYP2B6 was previously established by Ward, et al. (2003). EFV metabolism to 7-hydroxy-EFV catalyzed by CYP2A6 and to 8-14-dihydroxy-EFV catalyzed by CYP2B6 was previously established by Ogburn, et al. (2010).
Figure 1

A

![Blood Plasma Total](chart)

B

![Seminal Plasma Total and Protein-free](chart)

C

![Cerebrospinal Fluid Total](chart)
Figure 2

Graph 1: Intensity vs. Time (min) with peaks at 1.57 and 1.73.

Graph 2: Intensity vs. Time (min) with peaks at 1.56 and 1.73.

Graph 3: Intensity vs. Time (min) with a peak at 1.55.
Figure 3

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)
Figure 6

Efavirenz (EFV) → CYP2B6 → CYP1A1 → 8,14-dihydroxy EFV

CYP2A6

CYP2B6 → CYP1A1

8-hydroxy EFV → CYP2B6 → 7,8-dihydroxy EFV

CYP2C8

7-hydroxy EFV → CYP2B6 → dihydroxy EFV