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# Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV

Lucie Zelena[a], Josef Reznicek[b], Martina Ceckova[b], Hana Sklenarova[a,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Czechia
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Czechia

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ABSTRACT

Efavirenz is an antiretroviral drug used in the treatment of HIV-positive patients. A simple, fast and sensitive high-performance liquid chromatography (HPLC) method was developed in order to determine efavirenz in three types of samples provided from pharmacokinetic studies. The analysis took 5 min and was performed using a C18 analytical column (Discovery HS C18, 150 × 4.6 mm, particle size of 5 μm) in isocratic mode with a mobile phase containing acetonitrile and water (65:35, v/v), a flow rate of 1.6 mL $min^{-1}$, a sample volume of 10 μL and UV detection at 245 nm. Three different sample matrices (Opti-MEM medium, Krebs perfusion liquid and tissue lysate) and their treatment (dilution, SPE) were considered. The validated method was applied for the analysis of 805 real samples arising from *in vitro* transcellular transport assays and *in vivo* organ perfusion experiments in order to evaluate the interaction of efavirenz with ATP-dependent drug efflux transporters. The lack of interaction of efavirenz with ABCB1, ABCG2 and ABCC2 transporters as well as technical aspects of this analysis, including the adhesion of efavirenz to the plastic materials and the stability of the drug during different tissue lysis approaches are discussed.



## 1. Introduction

Efavirenz (Fig. 1) is an antiretroviral drug belonging to the group of non-nucleoside reverse transcriptase inhibitors (NNRTIs) used in the treatment of HIV-1 infections [1,2]. It represents a common part of first-line combined antiretroviral therapy (cART) when being co-administered usually with 1 or 2 other antiretroviral drugs [3,4].

Although the substance has already been approved by the U.S. Food and Drug Administration (FDA) in 1998 and a lot is known about its pharmacokinetic patterns [1,2], the development of a sensitive analytical method is important, especially for precise and fast assessment of pharmacokinetic drug-drug interactions (DDI) between efavirenz and other co-administered drugs. Drug transporters represent the major causative factor of pharmacokinetic DDI [6,7] and great attention is therefore currently being paid to employing *in vitro* transport experiments across transporter-overexpressing polarized cell monolayers or towards pharmacokinetic studies to evaluate transporter-mediated DDI *in situ* or *in vivo* [8,9]. The substrate specificity of efavirenz towards main ATP-dependent (ABC) drug efflux transporters was assessed [10–13], suggesting that efavirenz does not interact with ABCB1. Nevertheless, other transporters might be causative of efavirenz DDI.

Different sample types were collected and analysed in these assays using several distinct analytical approaches. Of these, two main approaches were used for the determination of efavirenz, namely, scintillation counting with radiolabelled standards [12] and HPLC analysis [10,11,13]. Cell suspensions, cell lysates, Wistar rat plasma and brain tissue were treated by extraction into pure acetonitrile and analysed using the HPLC method [10]. Samples taken from chambers of the Transwell system in transport studies across monolayers of intestinal Caco-2 cells were analysed by HPLC without any pre-treatment [11]. The transport of efavirenz across the intestine was evaluated using intestine sacs prepared from small intestine tissue, for which samples were taken from the inner medium and analysed by HPLC without any pre-treatment [13].

Several HPLC methods have been previously reported for the determination of efavirenz, alone [10,14–20] or in combination with other antiretroviral drugs [11,21–23] in human plasma using UV detection [10,11,14–16,18–23] or fluorescence detection after post-column derivatization [17]. These methods use common

* Corresponding author at: Charles University in Prague, Faculty of Pharmacy in Hradec Kralove, Department of Analytical Chemistry, Heyrovskeho 1203, 500 05 Hradec Kralove, Czechia.
*E-mail address:* sklenarova@faf.cuni.cz (H. Sklenarova).

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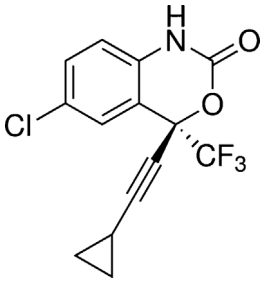


**Fig. 1.** Chemical structure of efavirenz [5].

sample pre-treatment procedures such as protein precipitation [14,15,19,20], liquid-liquid extraction (LLE) [11,16,17,21] and solid-phase extraction (SPE) [18,22,23].

Additionally, the objective was to apply the developed method for analysis of large series of samples using the HPLC method universal for tested matrices. The selection and optimization of HPLC conditions and sample pre-treatment is thoroughly discussed with respect to the HPLC column lifetime for the analysis of approximately 800 real samples. Additionally, a detailed pharmacokinetic study was performed of the interactions of efavirenz with different cell membrane transporters at levels of the cell monolayer, dually perfused placenta and its lysates.

The two major aims of this study were (i) to develop and validate a simple and universal analytical method for efavirenz determination in three complex sample matrices with low sample volumes and (ii) to verify the sensitivity of the analysis in three types of real samples obtained in transport assays across the MDCK cellular monolayer overexpressing ABC transporters, in organ perfusion and in tissue lysates.

## 2. Experimental

### 2.1. Chemicals and material

Efavirenz (EFV) and acetonitrile (ACN), CHROMASOLV® gradient grade for HPLC, were purchased from Sigma-Aldrich (Prague, Czech Republic). Ultra-pure water was obtained from a Milli-Q RG unit (Merck Millipore, Prague, Czech Republic).

Methylparaben, ethylparaben, propylparaben, butylparaben, dichlorophene, hexachlorophene, hydrocortisone, betamethasone, penicillin G sodium salt, propyphenazone, diclofenac sodium salt, β-estradiol 17-acetate and 17β-estradiol 3-methylether, tested as potential internal standards, were purchased from Sigma-Aldrich (Prague, Czech Republic).

Dulbecco's Modified Eagle's Medium (DMEM) and Opti-MEM I Reduced Serum Medium were purchased from Sigma-Aldrich (Prague, Czech Republic). Krebs perfusion liquid, an aqueous buffer containing sodium chloride (NaCl, 6.90 g $L^{-1}$), potassium chloride (KCl, 0.35 g $L^{-1}$), sodium bicarbonate ($NaHCO_3$, 2.10 g $L^{-1}$), sodium phosphate ($Na_2HPO_4$. $12H_2O$, 0.14 g $L^{-1}$), glucose (1.00 g $L^{-1}$), calcium chloride ($CaCl_2$. $2H_2O$, 0.21 g $L^{-1}$), magnesium chloride ($MgCl_2$. $6H_2O$, 0.12 g $L^{-1}$), heparin (1%, v/v) and dextran (10.00 g $L^{-1}$), was purchased from Sigma-Aldrich (Prague, Czech Republic) and prepared daily. SOLVABLE liquid (sodium hydroxide (≤2.5%), C11-15-secondary ethoxylated alcohols (2.5–10.0%) and C10-16-alkyldimethyl *N*-oxide amines (2.5–10%) in distilled water), tested for placenta lysis, were purchased from Perkin Elmer (Sigma-Aldrich, Prague, Czech Republic).

Whatman PTFE syringe filters (PTFE membrane, diameter of 4 mm, pore size of 0.2 μm) and Whatman PVDF filters (PVDF membrane, diameter of 4 mm, pore size of 0.2 μm) from GE Healthcare Life Science (BioTech s.r.o., Czech Republic), LUT Syringe Filter Nylon (nylon membrane, diameter of 4 mm, pore size of 0.2 μm) from Labicom (Czech Republic) and Microcon® Centrifugal Filter Ultracel® YM-10 (regenerated cellulose membrane YM-10, NMWCO 10 kDa) from Merck Millipore (Prague, Czech Republic) were tested for sample pre-treatment. A Sprout® Mini-Centrifuge (max. speed 6000 rpm, max. RCF 2000 × g) from Heathrow Scientific (USA) was used for sample filtration.

Solid-phase extraction cartridges, Discovery® DSC-18 SPE tube (sorbent weight of 500 mg, volume of 3 mL), and the Visiprep™ SPE Vacuum Manifold (Disposable Liner, 24-port model) were purchased from SUPELCO (Sigma-Aldrich, Prague, Czech Republic).

### 2.2. Cell lines and laboratory animals

MDCKII parental cell line (MDCK-PAR) and MDCKII cells stably transduced for expression of human transporters ABCB1, ABCG2 or ABCC2 (MDCK-ABCB1, MDCK-ABCG2 and MDCK-ABCC2, respectively) were provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All the MDCK cell lines were cultured in DMEM complete high-glucose medium with L-glutamine, supplemented with 10% fetal bovine serum.

Pregnant Wistar rats were purchased from MediTox Ltd. (Konarovice, Czech Republic) and maintained under 12/12 h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized by administering a dose of 40 mg/kg pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL, USA) into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

### 2.3. Chromatographic system and separation conditions

The chromatographic system, Nexera X2 (Shimadzu Corporation, Japan), consisted of two pumps (LC-30AD), two degassing units (DGU-20A5R), an autosampler (SIL-30AC), a column oven (CTO-20AC), a diode array detector (SPD-M30A) and a system controller (CBM-20A). The LabSolutions software was used for data acquisition and data analysis.

The analytical column Discovery® HS C18 (150 mm × 4.6 mm, particle size of 5 μm) with an OptiGuard 1 mm C18 guard column from SUPELCO (Sigma-Aldrich, Prague, Czech Republic) was used for separation of EFV from the matrix components and potential degradation products.

The mobile phase consisted of acetonitrile and ultra-pure water (65:35, v/v) with no pH adjustment. The injected sample volume was 10 μL. The UV detector was set at a wavelength of 245 nm. The sample analysis took 5 min and was performed in isocratic mode with a flow rate of 1.6 mL $min^{-1}$ and a temperature of 25 °C. A 10 min gradient washing step was included between perfusion sample measurements after every six samples.

### 2.4. Preparation of standard solutions

The EFV stock solution (45.00 mM) in DMSO was kept in a freezer (−20 °C). The EFV working solution (100.00 μM) was prepared by dilution of the EFV stock solution with Opti-MEM medium, Krebs perfusion liquid and the mobile phase for sample types A, B and C, respectively. EFV standard solutions (0.50–50.00 μM) were prepared by dilution of the EFV working solution by the Opti-MEM medium, Krebs perfusion liquid and the mobile phase for sample types A, B and C, respectively. The DMSO concentration was maintained at less than 0.02% (v/v) to prevent cytotoxic effects of DMSO