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Faculté de biologie
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Optimisation of antiretroviral therapy: pharmacokinetic and pharmacogenetic approaches

Thèse de Doctorat ès Sciences de la vie (PhD)

Présentée à la Faculté de Biologie et Médecine
de l'Université de Lausanne par:

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Christian Widmann

A Christophe

« L'aventure humaine, c'est de se poser des questions, d'explorer et de chercher »

Boris Cyrulnik

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Optimisation of antiretroviral therapy: pharmacokinetic and pharmacogenetic approaches

The improvement in antiretroviral therapy has transformed HIV infection from an inevitably fatal condition to a chronic, manageable disease. However, treatment failure and drug toxicity are frequent. Inadequate response to treatment is clearly multifactorial and, therefore, dosage individualisation based on demographic factors, genetic markers and measurement of total, free and/or cellular drug level may increase both drug efficacy and tolerability. Drug tolerability is certainly a major issue for a treatment that must be taken indefinitely.

The global objective of this thesis aimed at increasing our current understanding of pharmacokinetic (PK) and pharmacogenetic (PG) factors influencing the exposition to antiretroviral drugs (ARVs) in HIV-positive patients. In turn, this should provide us with a rational basis for antiviral treatment optimisation and drug dosage adjustment in HIV-positive patients. Patient's tailored antiretroviral regimen is likely to enhance treatment effectiveness and tolerability, enabling a better compliance over time, and hence reducing the probability of emergence of viral resistance and treatment failure.

To that endeavour, analytical methods for the measurement of total plasma, free and cellular concentrations of ARVs and some of their metabolites have been developed and validated using liquid chromatography coupled with tandem mass spectrometry. These assays have been applied for the monitoring of ARVs levels in various populations of HIV-positive patients. A clinical study has been initiated within the frame of the Mother and Child Swiss HIV Cohort Study to determine whether pregnancy influences the exposition to ARVs. Free and total plasma concentrations of lopinavir, atazanavir and nevirapine have been determined in pregnant women followed during the course of pregnancy, and were found not influenced to a clinically significant extent by pregnancy. Dosage adjustment for these drugs is therefore not required in pregnant women. In a study in treatment-experienced HIV-positive patients, the correlation between cellular and total plasma exposure to new antiretroviral drugs, notably the HIV integrase inhibitor raltegravir, has been determined. A good correlation was obtained between total and cellular levels of raltegravir, suggesting that monitoring of total levels are a satisfactory. However, significant inter-patient variability was observed in raltegravir cell accumulation which should prompt further investigations in patients failing under an integrase inhibitor-based regimen. The effectiveness of therapeutic drug monitoring (TDM) to guide efavirenz dose reduction in patients having concentrations above the recommended therapeutic range was evaluated in a prospective study. TDM-guided dosage adjustment of efavirenz was found feasible and safe, supporting the use of TDM in patients with efavirenz concentrations above therapeutic target. The impact of genetic polymorphisms of cytochromes P450 (CYP) 2B6, 2A6 and 3A4/5 on the PK of efavirenz and its metabolites was studied: a population PK model was built integrating both genetic and demographic covariates. Functional genetic variations in main (CYP2B6) and accessory (2A6, 3A4/5) metabolic pathways of efavirenz have an impact on efavirenz disposition, and may lead to extreme drug exposures. Dosage adjustment guided by TDM is thus required in those patients, according to the pharmacogenetic polymorphism.

Thus, we have demonstrated, using a comprehensive approach taking into account both PK and PG factors influencing ARVs exposure in HIV-infected patients, the feasibility of individualising antiretroviral therapy in various situations. Antiviral treatment optimisation is likely to increase long-term treatment success while reducing the occurrence of adverse drug reactions.

Optimisation de la thérapie antirétrovirale: approches pharmacocinétiques et pharmacogénétiques

Les progrès de la thérapie antirétrovirale ont transformé l'infection par le VIH d'une condition inévitablement fatale à une maladie chronique. En dépit de ce succès, l'échec thérapeutique et la toxicité médicamenteuse restent fréquents. Une réponse inadéquate au traitement est clairement multifactorielle et une individualisation de la posologie des médicaments qui se baserait sur les facteurs démographiques et génétiques des patients et sur les taux sanguins totaux, libres et/ou cellulaires des médicaments pourrait améliorer à la fois l'efficacité et la tolérance de la thérapie, cette dernière étant certainement un enjeu majeur pour un traitement qui se prend à vie.

L'objectif global de cette thèse était de mieux comprendre les facteurs pharmacocinétiques (PK) et pharmacogénétiques (PG) influençant l'exposition aux médicaments antirétroviraux (ARVs) nous offrant ainsi une base rationnelle pour l'optimisation du traitement antiviral et pour l'ajustement posologique des médicaments chez les patients VIH-positifs. Une thérapie antirétrovirale adaptée au patient est susceptible d'augmenter la probabilité d'efficacité et de tolérance à ce traitement, permettant ainsi une meilleure compliance à long terme, et réduisant le risque d'émergence de résistance et d'échec thérapeutique.

A cet effet, des méthodes de quantification des concentrations plasmatiques totales, libres et cellulaires des ARVs ainsi que de certains de leurs métabolites ont été développées et validées en utilisant la chromatographie liquide coupée à la spectrométrie de masse en tandem. Ces méthodes ont été appliquées pour la surveillance des taux d'ARVs dans diverses populations de patients HIV-positifs. Une étude clinique a été initiée dans le cadre de l'étude VIH Suisse de cohorte mère-enfant afin de déterminer si la grossesse influence la cinétique des ARVs. Les concentrations totales et libres du lopinavir, de l'atazanavir et de la névirapine ont été déterminées chez les femmes enceintes suivies pendant leur grossesse, et celles-ci ont été trouvées non influencées de manière cliniquement significative par la grossesse. Un ajustement posologique de ces ARVs n'est donc pas nécessaire chez les femmes enceintes. Lors d'une petite étude chez des patients HIV-positifs expérimentés, la corrélation entre l'exposition cellulaire et plasmatique des nouveaux ARVs, notamment le raltégravir, a été déterminée. Une bonne corrélation a été obtenue entre taux plasmatiques et cellulaires de raltégravir, suggérant que la surveillance des taux totaux est un substitut satisfaisant. Cependant, une importante variabilité inter-patient a été observée dans les ratios d'accumulation cellulaire du raltégravir, ce qui devrait encourager des investigations supplémentaires chez les patients en échec sous ce traitement. L'efficacité du suivi thérapeutique des médicaments (TDM) pour l'adaptation des taux d'efavirenz chez des patients avec des concentrations au-dessus de la cible thérapeutique recommandée a été évaluée lors d'une étude prospective. L'adaptation des doses d'efavirenz basée sur le TDM s'est montrée efficace et sûre, soutenant l'utilisation du TDM chez les patients avec concentrations hors cible thérapeutique. L'impact des polymorphismes génétiques des cytochromes P450 (*CYP*) 2B6, 2A6 et 3A4/5 sur la pharmacocinétique de l'efavirenz et de ces métabolites a été étudié : un modèle de PK de population intégrant les covariats génétiques et démographiques a été construit. Les variations génétiques fonctionnelles dans les voies de métabolisation principales (*CYP2B6*) et accessoires (*CYP2A6* et *3A4/5*) de l'efavirenz ont un impact sur sa disposition, et peuvent mener à des expositions extrêmes au médicament. Un ajustement des doses guidé par le TDM est donc recommandé chez ces patients, en accord avec les polymorphismes génétiques.

Ainsi, nous avons démonté qu'en utilisant une approche globale tenant compte à la fois des facteurs PK et PG influençant l'exposition aux ARVs chez les patients infectés, il est possible, si nécessaire, d'individualiser la thérapie antirétrovirale dans des situations diverses. L'optimisation du traitement antirétroviral contribue vraisemblablement à une meilleure efficacité thérapeutique à long terme tout en réduisant la survenue d'effets indésirables.

Optimisation de la thérapie antirétrovirale: approches pharmacocinétiques et pharmacogénétiques

Les progrès effectués dans le traitement de l'infection par le virus de l'immunodéficience humaine acquise (VIH) ont permis de transformer une affection mortelle en une maladie chronique traitable avec des médicaments de plus en plus efficaces. Malgré ce succès, un certain nombre de patients ne répondent pas de façon optimale à leur traitement et/ou souffrent d'effets indésirables médicamenteux entraînant de fréquentes modifications dans leur thérapie. Il a été possible de mettre en évidence que l'efficacité d'un traitement antirétroviral est dans la plupart des cas corrélée aux concentrations de médicaments mesurées dans le sang des patients. Cependant, le virus se réplique dans la cellule, et seule la fraction des médicaments non liée aux protéines du plasma sanguin peut entrer dans la cellule et exercer l'activité antirétrovirale au niveau cellulaire. Il existe par ailleurs une importante variabilité des concentrations sanguines de médicament chez des patients prenant pourtant la même dose de médicament. Cette variabilité peut être due à des facteurs démographiques et/ou génétiques susceptibles d'influencer la réponse au traitement antirétroviral.

Cette thèse a eu pour objectif de mieux comprendre les facteurs pharmacologiques et génétiques influençant l'efficacité et la toxicité des médicaments antirétroviraux, dans le but d'individualiser la thérapie antivirale et d'améliorer le suivi des patients HIV-positifs.

A cet effet, des méthodes de dosage très sensibles ont été développées pour permettre la quantification des médicaments antirétroviraux dans le sang et les cellules. Ces méthodes analytiques ont été appliquées dans le cadre de diverses études cliniques réalisées avec des patients. Une des études cliniques a recherché s'il y avait un impact des changements physiologiques liés à la grossesse sur les concentrations des médicaments antirétroviraux. Nous avons ainsi pu démontrer que la grossesse n'influençait pas de façon cliniquement significative le devenir des médicaments antirétroviraux chez les femmes enceintes HIV-positives. La posologie de médicaments ne devrait donc pas être modifiée dans cette population de patientes. Par ailleurs, d'autres études ont portés sur les variations génétiques des patients influençant l'activité enzymatique des protéines impliquées dans le métabolisme des médicaments antirétroviraux. Nous avons également étudié l'utilité d'une surveillance des concentrations de médicament (suivi thérapeutique) dans le sang des patients pour l'individualisation des traitements antiviraux. Il a été possible de mettre en évidence des relations significatives entre l'exposition aux médicaments antirétroviraux et l'existence chez les patients de certaines variations génétiques. Nos analyses ont également permis d'étudier les relations entre les concentrations dans le sang des patients et les taux mesurés dans les cellules où le virus HIV se réplique. De plus, la mesure des taux sanguins de médicaments antirétroviraux et leur interprétation a permis d'ajuster la posologie de médicaments chez les patients de façon efficace et sûre.

Ainsi, la complémentarité des connaissances pharmacologiques, génétiques et virales s'inscrit dans l'optique d'une stratégie globale de prise en charge du patient et vise à l'individualisation de la thérapie antirétrovirale en fonction des caractéristiques propres de chaque individu. Cette approche contribue ainsi à l'optimisation du traitement antirétroviral dans la perspective d'un succès du traitement à long terme tout en réduisant la probabilité des effets indésirables rencontrés.

Scientific communications

Some aspects of the present work have been published and presented in national and international congresses as oral or poster presentations.

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Abbreviations

AAG	α-1-acid glycoprotein
Alb	Albumin
ANOVA	Analysis of Variance
APV	Amprenavir
ART	Antiretroviral Therapy
ARV	Antiretroviral Drug
ATV	Atazanavir
AUC	Area Under the Curve
BID	Twice daily
C_{cell}	Cellular concentration
C_{max}	Maximum plasma concentration
C_{min}	Minimum plasma concentration
C_{tot}	Total plasma concentration
C_{trough}	Residual concentration
C_{free}	Free (unbound) plasma concentration
CCR	Cellular Chemokine Receptor
CPT	Cell Preparation Tube
CYP	Cytochrome P450
DRV	Darunavir
EFV	Efavirenz
ETV	Etravirine
FIs	Fusion Inhibitors
FPV	Fosamprenavir
F_u	Free fraction
GWAS	Genome-Wide Association Study
HAART	Highly Active Antiretroviral Therapy
HLA	Human Leukocyte Antigen
HSA	Human Serum Albumin
IDV	Indinavir
INIs	Integrase Inhibitors
LC	Liquid Chromatography
LPV	Lopinavir
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MVC	Maraviroc
NFV	Nelfinavir
NNRTIs	Non Nucleoside Reverse Transcriptase Inhibitors
NONMEM	NON-linear Mixed Effect Modeling
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NtRTIs	Nucleotide Reverse Transcriptase Inhibitors
NVP	Nevirapine
PBMCs	Peripheral Blood Mononuclear Cells
PD	Pharmacodynamics
P-gp	P-glycoprotein

PIs	Protease Inhibitors
PK	Pharmacokinetics
PG (ou PGx)	Pharmacogenetics
PopPK	Population Pharmacokinetics
QD	Once daily
RAL	Raltegravir
RTV	Ritonavir
SHCS	Swiss HIV Cohort Study
SQV	Saquinavir
TDM	Therapeutic Drug Monitoring
TPV	Tipranavir
UGT	UDP-glucuronosyltransferase

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PART I

INTRODUCTION

Chapter 1:
Optimising Antiretroviral Therapy

1.1. General introduction

The Human Immunodeficiency Virus (HIV) pandemic is undoubtedly the defining public-health crisis of our time. The number of people living with HIV worldwide continues to grow, reaching an estimated 33.4 million in 2008 (1). Today, there is no region untouched by this pandemic. As a response, the advent of antiretroviral therapy (ART), particularly the implementation of highly active antiretroviral treatment (HAART), has reduced HIV-related mortality and extended life expectancy for HIV patients and, at least in industrialised countries, has transformed Acquired Immunodeficiency Syndrome (AIDS) from an inevitably fatal condition to a chronic, manageable disease (2,3).

By the end of 2009, 26 years after the HIV has been isolated as the putative cause of AIDS, 25 antiretroviral compounds have been approved for clinical use (Figure 1). Moreover, these recent years, the introduction in the clinic of next generation non nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs), and potent novel antiretroviral drugs targeting the HIV entry and integration, has offered highly treatment-experienced patients new therapeutic options with more potent and hopefully more tolerable treatment regimens (4,5).

Despite the general success of combination therapy and the dramatic improvement in tolerability, safety and efficacy of therapy regimens (6-8), a significant percentage of patients still experience drug toxicity or HIV resistance, and do not reach adequate virologic suppression (9). Treatment failure is clearly multifactorial and, besides viral strain characteristics, it involves both host genetic background and environmental factors (i.e. drug interactions, dietary habits) influencing drug pharmacokinetics and treatment tolerability, impacting in turn the adherence to antiretroviral treatment.
In fact, the development of antiretroviral drugs has been conducted over a rather short period of time, limiting the capacity to fully define pharmacokinetics (PK) and the profile of medium – and long – term toxicity, and there is therefore still room for improvement in the use of antiretroviral drugs already on the market.

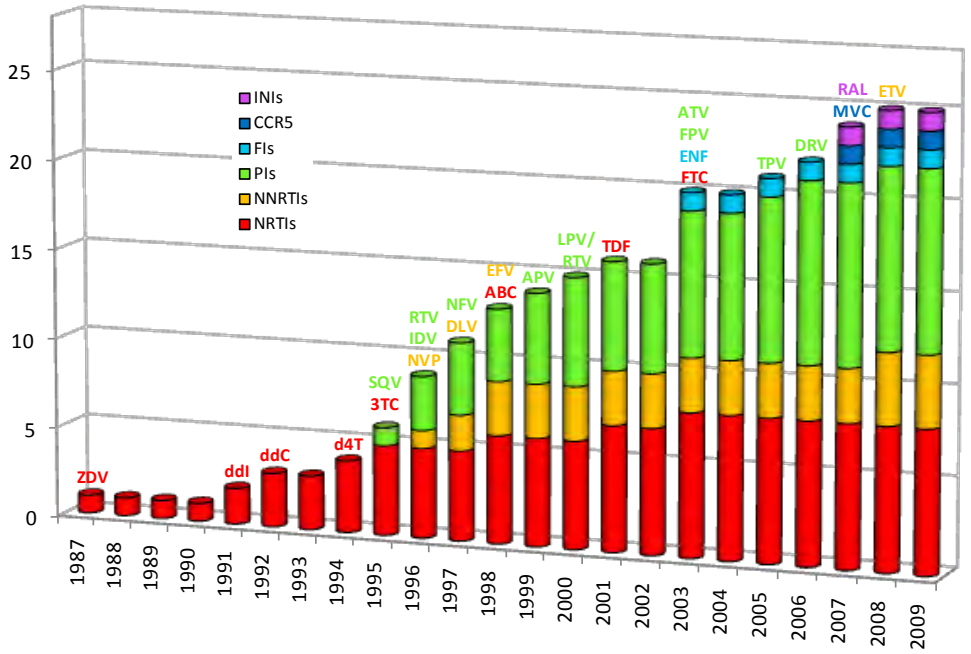

Figure 1: Approval of antiretroviral drugs by class.
NRTIs: nucleoside/nucleotide reverse transcriptase inhibitors; NNRTIs: non nucleoside reverse transcriptase inhibitors; PIs: protease inhibitors; FIs: fusion inhibitors; CCR5: CCR5 co-receptor antagonists; INIs: integrase inhibitors; ZDV: zidovudine; ddI: didanosine; ddC: zalcitabine; d4T: stavudine; 3TC: lamivudine; ABC: abacavir; TDF: tenofovir; FTC: emtricitabine; NVP: nevirapine; DLV: delavirdine; EFV: efavirenz; ETV: etravirine; SQV: saquinavir; IDV: indinavir; RTV: ritonavir; NFV: nelfinavir; APV: amprenavir; LPV: lopinavir; FPV: fosamprenavir; ATV: atazanavir; TPV: tipranavir; DRV: darunavir; ENF: enfuvirtide; MVC: maraviroc; RAL: raltegravir.

Moreover, the optimisation of current antiretroviral drugs' use is particularly important in the context of the innovative « Test and Treat » approach, a theoretical, albeit promising strategy recently published by scientists from the World Health Organisation (WHO) (10), which aims at eliminating HIV transmission and reducing the prevalence of HIV to less than 1% within 50 years worldwide by universal voluntary HIV testing and immediate treatment with ART for those who test HIV positive.

Typically, therapy individualisation based on demographic factors, genetic markers or measurement of circulating plasma concentrations are efficient tools that may enhance both the efficacy and the tolerability of antiretroviral drugs. Moreover, there is increasing evidence that patient treatment management could benefit from the emerging approaches integrating pharmacogenetics and applied clinical pharmacokinetics (11,12). Thus, individualised therapy could contribute to an important part to the improvement of HIV patient's clinical care and follow-up while sparing significant amounts of both patient suffering and healthcare costs.

In the perspective of an optimisation of ART, the relevance of dosage individualisation of antiretroviral drugs (i) in specific conditions, (ii) for special patient's populations, or (iii) for new drugs, is currently not known in many circumstances.

For instance, it is not clearly known to which extent the physiological changes due to pregnancy affect the efficacy and/or the safety of antiretroviral treatment for the mother and her child and necessitate antiretroviral drugs regimen adjustment. Indeed, during pregnancy, gastric pH tends to increase, and may alter the ionisation of molecules; the volume of distribution of drugs may be altered by plasma volume expansion; plasma albumin and α-1-acid glycoprotein (AAG) concentrations are decreased, partly due to haemodilution, thus potentially affecting protein binding; finally, renal excretion is generally increased, whereas hepatic drug metabolism may increase, decrease or remain unchanged (13,14). All these elements suggest significant variations of antiretroviral pharmacokinetics during pregnancy, which were evaluated by several studies previously reviewed (15,16). But whether these changes need to be really taken into consideration for Therapeutic Drug Monitoring (TDM) and dosage individualisation in HIV-positive pregnant women have not yet been formally proved. Moreover, as physiological changes may impact on total drug exposure, the determination of free drug concentration may therefore provide a more accurate indicator of "effective" drug exposure and eliminate a potential source of misinterpretations occurring with traditional TDM using total concentrations. Indeed, the official guidelines posted online on the FDA site propose dose adjustment during pregnancy (17) without much details on the rationale it is based on and how this should be formally done. A comprehensive study of the impact of pregnancy on free and total pharmacokinetics of antiretrovirals would be of great interest because this is, together with the better understanding of placental transfer of antiretroviral drugs (18), a critical issue in the context of the prevention and reduction of mother-to-child HIV transmission (19-21).

Alternately, as efavirenz (EFV) is currently administered at the usual fixed dose of 600 mg once daily despite the marked interindividual variability in exposure, some patients present excessive plasma concentrations of the drug, and are at high risk of experiencing neuropsychological side effects (22,23). Besides gender and ethnicity (11,24), genetic polymorphisms of cytochrome P450 2B6 (*CYP2B6*), the principal metabolic pathway of EFV (25), explain to a large extent the interindividual variability in exposure (26-29). In this specific population, the feasibility of efavirenz dosage individualisation by Therapeutic Drug Monitoring (TDM) to bring plasma concentrations

within the recommended therapeutic range, and its impact on viral control, have never been formally assessed in a controlled clinical study. Furthermore, comparing drug dosage reduction based on TDM with dosage reduction predicted by genetic testing, have never been addressed.

Indeed, a general approach combining genotypic and phenotypic information could help to predict EFV exposure and individualise treatment regimen in order to reach "ideal" concentrations so as to prevent excessive exposure and toxicity. This approach is possible with the development of a pharmacogenetic-based population pharmacokinetic model that integrates both the information on genetic polymorphisms of *CYP2B6* and the demographic factors influencing EFV drug exposure. Besides this interindividual variability in EFV exposure explained to a large extent by genetic variations in *CYP2B6*, there is still a remaining unexplained variability, in particular among individuals with impaired CYP2B6 function (27), which could be due to polymorphisms of *CYP2A6* and *3A4/5*, the accessory metabolic pathways of EFV. The clinical importance of functional polymorphisms in *CYP2A6* and its impact on metabolites profile have not yet been addressed, and this was completed via an *in vivo* metabolite profiling study.

Although intracellular concentrations of current PIs and NNRTIs have been widely described and correlated to various extent to total plasma levels (30-33), cellular PK for more recent classes of antiretroviral drugs acting at new HIV targets, such as raltegravir and maraviroc, as well as of the next-generation PI darunavir and NNRTI etravirine, remains a largely unexplored field of investigation. Thus, it is not yet known whether plasma concentrations reflect intracellular levels at the site of pharmacological activity for these new agents. For the first HIV integrase inhibitor raltegravir (RAL) particularly, relationships between PK and pharmacodynamics (PD) have been difficult to established using RAL total plasma concentrations (34), suggesting that other markers of RAL exposure may better predict its pharmacological activity in patients. To date, only one study (35) has tried to quantify RAL in peripheral blood mononuclear cells (PBMCs) from patients but have found no measurable levels of RAL in patient's cells with the methodology used (36). However, since only the fraction reaching the cellular compartment is expected to exert its antiviral action, information on RAL cellular disposition would allow ascertaining whether Therapeutic Drug Monitoring used for dosage individualisation using drugs levels measurement in plasma is a valid surrogate of RAL concentration in cells where HIV replicates.

1.2. Objectives of the thesis

The overall objective of this thesis is to better understand pharmacokinetic and pharmacogenetic factors influencing current and newly introduced antiretroviral drugs exposure in HIV-positive patients.

To that endeavour, the concrete goals of this work were:

- to develop and validate analytical methods for quantification of total and free plasma levels and intracellular concentrations of antiretroviral drugs and some of their metabolites (Chapter 2),
- to study total and free plasma concentrations of antiretroviral drugs in the general HIV patient population, but also during pregnancy and postpartum, and to perform a population pharmacokinetic analysis in this specific condition (Chapter 4),
- to assess the efficacy of efavirenz dose reduction guided by Therapeutic Drug Monitoring in patients with plasma concentrations above the usual recommended therapeutic range (Chapter 5),
- to investigate the influence of cytochrome P450 genetic polymorphisms on pharmacokinetics of efavirenz and some of its metabolites (Chapter 5),
- and to determine the relationship between total plasma and intracellular concentrations for raltegravir and other new drugs (Chapter 6).

This knowledge is expected to contribute to improve treatment individualisation, thereby optimising antiretroviral therapy in HIV-infected patients.

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PART II

METHODS

Chapter 2:
Tools to Optimise ARV Therapy

The strategy developed during this thesis aimed at increasing our current understanding of the complex gene-environmental interplay influencing toxicity and efficacy of antiretroviral treatment at the patient and the population levels, aiming at offering new possibilities for improving the long term tolerability ad response to ARV treatments. This comprehensive approach necessitated the development and validation of various methods encompassing various aspects of bioanalysis, pharmacokinetics and pharmacogenetics.

This chapter will describe the different tools that have been elaborated and applied, alone or in combination during this present work, for the ultimate goal of patients' therapy optimisation.

First, new analytical assays developed to measure total, free, and intracellular concentrations of current and new drugs will be presented, as well as innovative methods to establish metabolite profiling of drugs. Secondly, concepts of Therapeutic Drug Monitoring (TDM), population pharmacokinetics modelling and analyses based on Bayesian approach will also be discussed. Finally, some aspects of pharmacogenetics relevant to this work will be briefly reviewed.

2.1. Total drug concentration

Total drug concentration corresponds to a final, composite phenotypic trait, the consequence of pharmacogenetic and non-genetic factors influencing drug transport and metabolism. Thus, for many therapeutic agents, the precise and accurate determination of total drug levels is the first essential component of the monitoring of antiretroviral therapy.

It is important to provide for clinical services and research projects accurate and precise analytical methods not only for the most frequently prescribed antiretroviral drugs, but also for the more recent agents approved by the FDA and increasingly prescribed to patients.
In that perspective, we have set-up and validated a high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of the four recent antiretroviral drugs: raltegravir (and its glucuronide), maraviroc, darunavir and etravirine.

This analytical method proved to be suitable for monitoring new antiretroviral drugs and for determining interactions in new regimens for which clinicians have limited experience, and which may modify drug systemic disposition to an extent likely to impair virologic response. This methodology is therefore able to respond to the clinical demands for monitoring novel drugs administered in combination, often as salvage therapy, to heavily pre-treated patients having experienced treatment failure, and for whom exposure, tolerance and adherence assessments are critical issues.

A LC–TANDEM MS ASSAY FOR THE SIMULTANEOUS MEASUREMENT OF NEW ANTIRETROVIRAL AGENTS: RALTEGRAVIR, MARAVIROC, DARUNAVIR, AND ETRAVIRINE

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J Chrom B Analyt Technol Biomed Life Sci 2009; 877: 1057-1069

pdf version: Appendix 2.1.

2.1.1. Abstract

Raltegravir (RAL), maraviroc (MVC), darunavir (DRV),and etravirine (ETV) are new antiretroviral agents with significant potential for drug interactions. This work describes a sensitive and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of plasma drug levels.

Single step extraction of RAL, MVC, DRV, ETV and RTV from plasma (100 μl) is performed by protein precipitation using 600 μl of acetonitrile, after the addition of 100 μl darunavir-d_9 (DRV-d_9) at 1000 ng/ml in MeOH/H_2O 50/50 as internal standard (I.S.). The mixture is vortexed, sonicated for 10 min, vortex-mixed again and centrifuged. An aliquot of supernatant (150 μl) is diluted 1:1 with a mixture of 20mM ammonium acetate/MeOH 40/60 and 10 μl is injected onto a 2.1 x 50 mm Waters Atlantis™ - dC18 3μm analytical column.

Chromatographic separations are performed using a gradient program with 2mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytes quantification is performed by electrospray ionisation-triple quadrupole mass spectrometry using the selected reaction monitoring detection in the positive mode.

The method has been validated over the clinically relevant concentrations ranging from 12.5-5'000 ng/ml, 2.5-1'000 ng/ml, 25-10'000 ng/ml, 10-4'000 ng/ml, and 5-2'000 ng/ml, for RAL, MRV, DRV, ETV and RTV, respectively. The extraction recovery for all antiretroviral drugs is always above 91%. The method is precise, with mean inter-day CV% within 5.1-9.8 %, and accurate (range of inter-day deviation from nominal values –3.3 to +5.1 %). In addition our method enables the simultaneous assessment of raltegravir-glucuronide. This is the first analytical method allowing the simultaneous assay of antiretroviral agents targeted to four different steps of HIV replication. The proposed method is suitable for the Therapeutic Drug Monitoring Service of these new regimen combinations administered as salvage therapy to patients having experienced treatment failure, and for whom exposure, tolerance and adherence assessments are critical.

2.1.2. Introduction

Therapeutic interventions in HIV infection have been up to now mainly directed towards two viral enzymes, reverse transcriptase and protease. Despite the clinical efficacy of multiple drug combination treatments generally observed with current HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), resistant HIV strains appearance continuously necessitates alternative antiretroviral regimens.

Development of new agents has expanded the number of available molecules in the currently available classes as well as in novel classes. The new agents are of particular interest for treatment-experienced patients with multidrug resistant HIV for whom therapeutic options are limited.

Raltegravir (RAL; Isentress®) is the first member of the long-anticipated class of HIV integrase inhibitors that has reached the final stage of clinical development. Raltegravir has antiviral activity in both naïve (1,2) and antiretroviral-experienced patients (3-5) with so far a mostly favourable safety profile. Alternately, new therapeutic approaches directed to host, not viral, targets are also emerging: maraviroc (MVC; Celsentri®, Selzentry®) is a chemokine CCR5 co-receptor antagonist (6-9) that is used at present, in experienced, R5-tropic HIV infected patients for whom previous antiretroviral regimens have failed.

Darunavir (DRV, TMC114; Prezista®) is a PI of the latest generation characterized by a good activity against multi-drug resistant viral isolates and is used in association with low-dose ritonavir, in experienced patients with limited treatment options (10,11).

Finally, etravirine (ETV, TMC125; Intelence®) is a novel chemical class of NNRTIs with potent *in vitro* activity against both wild-type and some resistant HIV (notably the K103N mutants) to currently approved NNRTIs.

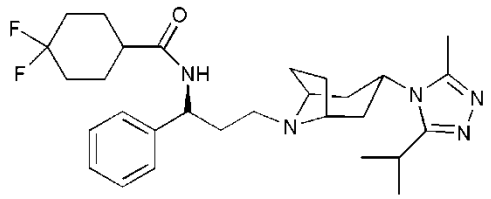
Various combination regimen with darunavir, etravirine and/or raltegravir are currently being studied: the DUET studies (darunavir and etravirine) (12-15), and more recently, the TRIO study (darunavir plus etravirine and raltegravir) with promising results (16).

Among these four drug classes, Therapeutic Drug Monitoring (TDM) of NNRTIs and PIs yields some clinical benefit for the management of antiretroviral treatment (17). Whether TDM is also beneficial for the new classes of integrase inhibitors and CCR5 antagonists remains to be established, but can be anticipated considering their metabolic pathways and the drug interaction potential of complex multiple agent-based regimen. The HIV integrase inhibitor raltegravir is primarily metabolized by uridine-5'-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) (18), an enzyme that is characterized by genetic polymorphism and that can be also inhibited by the PI atazanavir (19). The CCR5 antagonist maraviroc is a P-glycoprotein substrate and is extensively metabolized by the CYP3A isoform pathways. As a consequence, maraviroc plasma Area-Under-the-Curves (AUCs) are enhanced in the presence of potent inhibitors (ritonavir) and decreased in the presence of CYP3A inducers such as the NNRTIs efavirenz or etravirine, requiring maraviroc dosage adjustment (20). Finally, the novel PI darunavir, mainly metabolized by CYP3A4 isoform, is used in association with the CYP3A

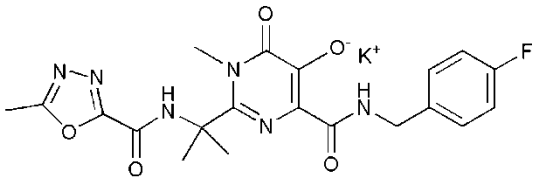
inhibitor RTV as pharmaco-enhancer and DRV/r combination is therefore at similar risk of the various drug interactions potential as most regimens with boosted PIs.
All these new agents have therefore the potential for significant reciprocal drug-drug interactions not to mention those with other antiretroviral medications, adding to the challenges of constructing long-term effective combination antiretroviral regimens. To that endeavour careful monitoring of drug exposure of these new drugs seems to be an important component of patients follow-up.

To date, several methods have been published for the assay of raltegravir by HPLC coupled to fluorescence (21) or photodiode array detection, with other antiretroviral drugs (22), or by LC-MS/MS alone (23,24) or together with its glucuronide metabolite (18,25). Reports have also been recently published describing chromatographic methods for the assay of darunavir by HPLC-UV (22,26) or by LC-MS/MS, together with other antiretroviral drugs (27-30). Assays for maraviroc have been described, as part of pre-clinical and clinical studies, without however much details on the validation of the analytical method (31-35). Finally, even though analytical methods have previously been developed for etravirine within the frame of drug interactions studies (14,15,36), only one formal validation of an HPLC method using off-line solid-phase extraction, followed by reversed-phase chromatography and UV photodiode array detection has been reported so far for the measurement of etravirine, along with raltegravir, darunavir and several other antiretroviral agents (22). This method, however, was unable to achieve a chromatographic separation for darunavir and amprenavir which co-eluted as a single peak. Recently, a LC-tandem MS has been proposed for the determination of etravirine in various biological matrices (37).
Complex salvage therapy regimens given to failing patients often combine raltegravir with boosted darunavir and etravirine, and possibly maraviroc in CCR5-tropic viral infection. Such regimens mandate therefore an analytical method enabling the unambiguous measurement of these various antiretroviral drugs altogether.
In this report, we therefore describe the development and validation of a straightforward analytical method for the simultaneous analysis in plasma of the four most recently licensed new antiretroviral agents raltegravir, maraviroc, darunavir, etravirine (chemical structures in Figure 1) and ritonavir by liquid chromatography coupled with tandem triple quadrupole mass spectrometry detection. This method is characterized by a very low limit of quantification, below the clinically relevant range of concentrations encountered in patients. Interestingly, our method enables the simultaneous determination of raltegravir-glucuronide, giving some insight of the role of

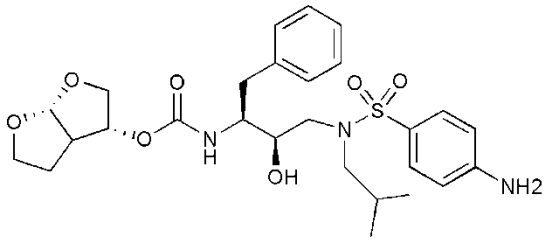
the UGT1A1-mediated metabolism of raltegravir. This method is currently applied in our TDM Service for patients follow-up and for clinical research projects within the frame of the Swiss HIV Cohort Study.



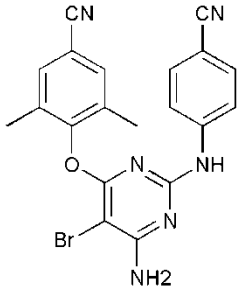
maraviroc (Selzentry®)



raltegravir (Isentress®, MK-0518)



darunavir (Prezista®, TMC114)



etravirine (Intelence®, TMC125)

Figure 1: Chemical structures of new antiretroviral drugs

2.1.3. Experimental

2.1.3.1. Chemicals and reagents

Raltegravir potassium (RAL) was kindly provided by Merck (Rahway, NJ, USA), maraviroc base (MVC) by Pfizer (New York, USA), darunavir ethanolate (DRV) and etravirine base (ETV) by Tibotec (Mechelen, Belgium) and ritonavir base (RTV) by Abbott (Baar, Switzerland). Darunavir-d_9 (DRV-d_9) purchased from LGC Prochem (Molsheim, France) was used for internal standard (I.S.) for the assay.

Acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH), all LiChrosolv® grade, and 100% formic acid were purchased from Fluka (Buchs, Switzerland). All other chemicals (including ammonium acetate) were of analytical grade and used as received. Ultrapure

water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA).

Blank plasma used for the assessment of matrix effect and for the preparation of calibration and control samples were isolated (1850 g, 10 min , +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from blood withdrawn from patients with Vaquez Disease.

2.1.3.2. Equipment

The high-performance liquid chromatography system involved a Rheos 2200 binary pump (Flux Instruments, Basel, Switzerland) equipped with an online degasser and a temperature-controlled 324 vial autosampler maintained at +10°C (CTC Analytics AG, Zwingen, Switzerland). Separations were done on a 2.1 x 50 mm Waters Atlantis™ - dC18 3μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 25°C (Croco-Cil, Cluzeau Info Laboratory, Courbevoie, France). The chromatographic system was coupled to a triple-stage quadrupole mass spectrometer (TSQ Quantum) from Thermo Fisher Scientific (Waltham, MA) equipped with an Ion Max electrospray ionization interface and operated of Xcalibur software package (version 2.0) (ThermoQuest, Thermo Fisher Scientific Inc, Waltham, MA).

2.1.3.3. Solutions

Mobile phase solutions

Solution A consisted of 2mM ammonium acetate in ultrapure water containing 0.1% formic acid (FA) (pH 2.8). Solution B consisted of 0.1% formic acid in MeCN. Solvents were regularly prepared prior to each series of analysis.

Internal standard, calibration standard and quality control (QCs) solutions

A stock solution of the internal standard (I.S.) darunavir-d_9 (DRV-d_9) at 1 mg/ml (in MeOH) was diluted with MeOH/H_2O 50/50 (vol/vol) to obtain a working solution at 1000 ng/mL.

Stock solutions of MVC (0.2 mg/ml in MeOH), DRV and RTV (1 mg/ml in MeOH), ETV (1 mg/ml in EtOH + FA, to pH 4.2) and RAL (2 mg/ml in MeOH/H_2O 50/50) were diluted with MeOH/H_2O 50/50 for the preparation of working solutions at concentrations ranging from 50 to 200'000 ng/ml (depending on the antiretroviral drug). These working solutions were diluted 1:20 with blank plasma to obtain the calibration samples

ranging from 2.5 to 10'000 ng/ml and the corresponding three quality control samples (low (L), medium (M) and high (H) QCs) from 25 to 7'500 ng/ml (Table 1). All solutions were prepared according to the recommendations on bioanalytical methods validation stating that the total added volume must be ≤ 10 % of the biological sample volume (38,39). The calibration and QCs samples were stored as 1 ml-aliquots at -20 °C prior to analysis.

Table 1: Concentration of working solutions and preparation of calibration and QC samples

Drug	Stock solution solvent	Stock solution concentration	Working solution concentration (obtained by dilution of stock solution with MeOH/H_2O 1:1)	Calibration range (obtained by dilution of working solution with plasma 1:20)	QCs controls
Darunavir (DRV)	MeOH	1 mg/ml	500 - 200'000 ng/ml	25 - 10'000 ng/ml	250; 1250; 7500 ng/ml
Etravirine (ETV)	EtOH	1 mg/ml	200 - 80'000 ng/ml	10 - 4'000 ng/ml	100; 500; 3000 ng/ml
Maraviroc (MVC)	MeOH	0.2 mg/ml	50 - 20'000 ng/ml	2.5 - 1'000 ng/ml	25; 125; 750 ng/ml
Raltegravir (RAL)	MeOH/H_2O 50/50	2 mg/ml	250 - 100'000 ng/ml	12.5 - 5'000 ng/ml	125, 625; 3750 ng/ml
Ritonavir (RTV)	MeOH	1 mg/ml	100 - 40'000 ng/ml	5 - 2'000 ng/ml	50; 250; 1500 ng/ml

2.1.3.4. LC-MS/MS conditions

The mobile phase was delivered using a stepwise gradient elution program: 2% of B at 0 min, 30% of B at 3.2 min, 100% of B at 10.0 min, with a flow rate of 0.3 ml/min. The second part of the run includes an intensive rinsing (100% of B at 10.1 min with 0.5 ml/min) for 5 min and a re-equilibration step to the initial solvent composition up to 20 min (at 15.1 min with 0.4 ml/min and at 18.0 min with 0.3 ml/min). The thermostated column heater was set at 25 °C and the autosampler was maintained at 10°C. The injection volume was 10 μl.

The mass spectrometer was operated with the electrospray ionisation source Ion Max in the positive mode. Samples were analysed via selected reaction monitoring (SRM) detection mode, employing the transition of the $[M+H]^+$ precursor ions to product ions. The selected *m/z* transitions and the collision energy (CE) for each analyte are reported in the Table 2.

The determination of optimal potential settings and MS/MS transitions were chosen by direct infusion into the MS/MS detector of a MeOH/H_2O 50/50 solution of all drugs at a concentration of 1 μg/ml. The first (Q1) and third (Q3) quadrupoles were set at 1 amu mass resolution (full-width half-maximum = 0.7), except for ETV, for which the full-width half-maximum was 1.5 (= 2.1 amu mass resolution). Scan time and scan width

were 0.04 sec and 1.0 *m*/*z*, respectively, and each chromatographic peak was the result of around 30 scans.

The ionisation conditions were as follows: the capillary temperature was set at 350 °C. The ESI spray voltage was set at 4 kV, the source induced dissociation was set at 10V. The sheath and auxiliary gas (nitrogen) flow-rate was set at 35 and 10 (arbitrary units), respectively. The tube lens voltages range from 67 to 123V and the Q2 collision gas (argon) pressure was 1mTorr.

Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur LC-Quan software package.

Table 2: Instrument method for the LC-MS/MS analysis for RAL, MVC, DRV, RTV and ETV with DRV-d_9 as internal standard

Drug	Parent m(m/z)	Product (m/z)	CE (eV)	Tube lens (V)	Typical RT (min)
Darunavir (DRV)	548.3	392.0	25	75	7.4
Etravirine (ETV)	434.9	303.9	49	111	9
Maraviroc (MVC)	514.3	280.0	40	87	5.6
Raltegravir (RAL)	445.1	361.0	27	84	6.7
Ritonavir (RTV)	721.4	296.2	26	123	8.2
Darunavir-d_9 (DRV-d_9)	557.3	401.2	25	67	7.4

CE = collision energy; mean RT = retention time; MS acquisition time (min) = 15.25; Q2 collision gas pressure (mTorr) = 1.00

2.1.3.5. Plasma sample preparation

Selection of the reconstitution solvent

During the initial development of the method, the following solvents were evaluated for the reconstitution of the plasma extract supernatant: 20 mM ammonium acetate/MeOH 50/50, 40/60 and 30/70. Among the solvent mixtures tested, the 40/60 provided the best chromatographic behaviour and peaks area intensity overall for RAL, MVC, DRV, RTV, ETV and the I.S. DRV-d_9, and was consequently used thereafter throughout the method.

Extraction procedure

A 100 µl aliquot of plasma sample was mixed with 100 µl of I.S. solution (1000 ng/ml DRV-d_9 in MeOH/H_2O 50/50) and 600 µl of MeCN, vortexed and sonicated for 10 minutes (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was again vortex-mixed and finally centrifuged at +4°C for 10 minutes at 20'000 g (14'000 rpm) on

a Hettich Benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). An 150 μl-aliquot of the supernatant was diluted 1:1 with 20 mM ammonium acetate/MeOH 40/60 into 500 μl glass HPLC microvials and after secure closing with crimp seals finally vortexed. A volume of 10 μl was used for LC-MS/MS analysis.

2.1.3.6. Quantification

Calibration curves

Quantitative analysis of the five antiretroviral drugs was performed using the internal standard method. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared with plasma isolated from outdated blood transfusion bags.

A seven-point calibration standard curve was calculated and fitted either by $1/x$ or $1/x^2$ weighted quadratic regression, or quadratic log-log regression, when appropriate, of the peak area ratios (drug peak area/ I.S. peak area) versus concentrations. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The calibration for the five antiretroviral drugs was established over the range reported in Table 1, so as to cover the range of clinically relevant concentrations expected in patients. Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0) (ThermoQuest, Thermo Fischer Scientific Inc, Waltham, MA).

2.1.3.7. Analytical method validation

The method validation procedure was based on the recommendations published on-line by the FDA (38) as well as on the updated recommendations of the Conference Report of the Washington Conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies" (39).

Accuracy and precision

Replicate analysis (n=6) of QC samples at the three concentrations (low (L), medium (M), high (H)) (Table 3) were used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by repeated analysis performed on six different occasions (Table 3). The concentration in each sample was determined using calibration standards prepared on the same day. The

precision was calculated as the coefficient of variation (CV %) within a single run (intra-assay) and between different assays (inter-assay), and the accuracy as the percentage of deviation between nominal and measured concentrations.

Table 3: Precision and accuracy of the assay for the five antiretroviral drugs in human plasma with QC samples at low, medium and high concentrations

	Nominal conc. [ng/ml]	INTER-ASSAY (n=6) Concentration found [ng/ml]	s.d. ±	Precision C.V. %	Accuracy Biais %	INTRA-ASSAY (n=6) Concentration found [ng/ml]	s.d. ±	Precision C.V. %	Accuracy Biais %
Darunavir	250	253.6	18.2	7.2	1.4	257.6	18.8	7.3	3.0
	1250	1313.5	67.7	5.2	5.1	1261.7	85.2	6.8	0.9
	7500	7400.6	482.2	6.5	-1.3	7715.6	355.2	4.6	2.9
Etravirine	100	103.5	8.3	8.1	3.5	94.1	6.3	6.7	-5.9
	500	483.3	33.2	6.9	-3.3	500.1	27.6	5.5	0.0
	3000	2945.5	225.0	7.6	-1.8	3291.2	108.6	3.3	9.7
Maraviroc	25	26.3	2.4	9.2	5.0	24.5	1.6	6.4	-2.1
	125	122.5	6.3	5.1	-2.0	124.7	9.0	7.2	-0.3
	750	727.6	62.3	8.6	-3.0	804.8	28.1	3.5	7.3
Raltegravir	125	129.3	12.6	9.8	3.5	122.9	8.7	7.1	-1.7
	625	604.7	35.8	5.9	-3.3	617.8	29.3	4.7	-1.2
	3750	3673.8	302.6	8.2	-2.0	3697.2	162.4	4.4	-1.4
Ritonavir	50	51.5	4.2	8.1	2.9	50.5	4.5	8.9	1.0
	250	245.9	17.0	6.9	-1.6	245.8	16.4	6.7	-1.7
	1500	1484.2	143.3	9.7	-1.1	1552.0	136.8	8.8	3.5

During the routine analysis of patient samples, control samples at each QC concentration levels were assayed. The analytical series were considered valid and accepted only if the percentage of deviation (bias) between nominal and back-calculated (experimental) concentrations for each calibration level and quality control samples were less than ± 15%, and less than ± 20% at the limit of quantification (defined as the lowest calibrator).

Limit of quantification and limit of detection

Serial dilutions (1/2, 1/5, 1/10, 1/16, 1/25, 1/50) of the lowest QC sample were analysed in triplicates. The lowest limit of quantification (LLOQ) for each drug analysed was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the documents mentioned above, recommending that the deviation between measured and nominal concentration should not deviate more than ± 20%. Calibration curves were established with calibration standards including either one or the other of dilution samples. The LLOQ

concentrations were finally selected as the lowest levels of the calibration curves with a bias and CV% below ± 20%.

A second set of dilutions (1/10, 1/20, 1/40, 1/50, 1/100, 1/200, 1/400) of the lowest QC sample and a blank plasma extract were analysed to determine the limit of detection (LOD), defined as the concentration that produced a signal three times above the noise level of a blank preparation.

Stability of antiretroviral drugs

Stability studies of RAL, MVC, DRV, ETV and RTV included:

a) Stability of plasma spiked with these antiretroviral drugs kept at room temperature (RT) and in the fridge at +4°C: the concentrations were measured immediately after preparation and after being left at room temperature (RT) and at +4°C up to 48 h. Antiretroviral drugs concentrations variations were expressed as a percentage of the nominal concentration.
b) Stability of plasma samples after multiple freeze-thaw cycles: QC samples at L, M and H levels of antiretroviral drugs underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at room temperature for 1 h and were subsequently refrozen during 2 h. Antiretroviral drugs concentrations were measured in aliquots from the three consecutive freeze-thaw cycles. The variations of concentrations were expressed as a percentage of the initial concentration measured at the beginning of the stability study.
c) Stability of plasma samples kept frozen at –20°C: The response of freshly prepared plasma calibration and QC samples was compared to those of frozen calibration and QCs samples stored during 6 weeks at –20°C.

Matrix effect and recovery

In the initial step of method validation, the matrix effect was examined qualitatively by the simultaneous post-column infusion of the five antiretroviral drugs and the I.S. into the MS/MS detector during the chromatographic analysis of blank plasma extracts. The standard solution of all analytes at 100 ng/ml was infused at a flow-rate of 10 µl/min during the chromatographic analysis of 6 blank plasma extracts from 6 different sources. The chromatographic signals of each selected MS/MS transition were examined to ascertain that no major signal perturbation (drift or shift) of the MS/MS signal was present at the analyte's retention time.

Subsequently, the quantitative determination of the matrix effect, and the determination of its variability were also assessed. Three series of QC samples at L, M and H concentration in duplicates were prepared as followed:

(A) Pure standard solutions of antiretroviral drugs, subjected to the same extraction/buffer dilution as for plasma, directly injected onto column;

(B) Blank plasma extract samples from 6 different sources, spiked with antiretroviral drugs and I.S *after* extraction;

(C) Plasma samples from 6 different sources (same as in B) spiked with QC standard solutions and I.S. *before* extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effect) was assessed by comparing the absolute peak areas of analytes either solubilised in extraction/buffer medium (A), or added after (B) and before (C) extraction of 6 different batches of plasma, based on the recommendations proposed by Matuszewski *et al.* (40,41).

The *extraction yield* of antiretroviral drugs and I.S. was calculated as the absolute peak-area response in processed plasma samples spiked with drugs *before* extraction (C), expressed as the percentage of the response of the same amount of drugs added into blank plasma extracts *after* the extraction procedure (B) (C/B ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of analytes added into blank plasma extracts *after* the extraction procedure (B) to the peak areas of pure analytes solubilised in extraction/buffer medium (A) (B/A ratio in %). The *overall recovery* of antiretroviral drugs and I.S. was calculated as the ratio of absolute peak-area response of antiretroviral drugs either in processed plasma samples spiked with drugs *before* extraction (C) to the peak areas of analytes solubilised in extraction/buffer medium (A) (C/A ratio in %).

Influence of plasma matrix variability on raltegravir-glucuronide/raltegravir ratios values

Raltegravir-glucuronide pure standard was not available to us and our method allows therefore only a relative (rather than absolute) measurement of raltegravir-glucuronide levels in patients, assessed by the raltegravir-glucuronide/raltegravir (RAL-gln/RAL) ratio. Whether the variability in patients' plasma matrix significantly influences the efficiency of raltegravir-glucuronide in-source dissociation, and hence the (RAL-gln/RAL) ratios values, was verified with the following experiments: plasma from HIV patients on raltegravir with high (>2.5), elevated (>1.0) and low (<0.3) RAL-gln/RAL ratios were diluted 1:2 with blank plasma from 6 different sources (healthy volunteers, Vaquez patients). The RAL-gln/RAL ratios measured in these patient samples before and after

the addition of various plasma matrices were compared. In fact, this comes to study the matrix effect variability at the retention time of RAL-gln, similarly to what is done for the other drugs (see Table 6).

Dilution effect

Some patient samples were found to contain drug concentrations exceeding the high level of the calibration curve (see Table 1). To ascertain whether the dilution of these samples prior to a subsequent analysis could affect the accuracy of the drug determination, a blank plasma sample was spiked with antiretroviral drugs at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in triplicate after a 5-fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

2.1.4. Results

2.1.4.1. Chromatograms

The proposed method enables the simultaneous quantification of the latest generation antiretroviral drugs (RAL, MVC, DRV, ETV) and RTV by liquid chromatography coupled with tandem MS/MS. Typical chromatographic profiles of calibration and QC samples containing RAL, MVC, DRV, ETV and RTV at concentrations corresponding to the lowest calibration level (i.e. corresponding to LLOQ) and to the highest QC level are shown in Figure 2a and b, respectively, in the positive mode, using the Selected Reaction Monitoring Mode (SRM) and the proposed gradient program (§ 2.1.3.4). The respective retention times of antiretroviral drugs and the I.S. DRV-d_9 are reported in Table 2. The separation is satisfactory between 5.5 and 9.0 minutes for all considered analytes.

Though all antiretroviral drugs and I.S. were eluted within 10 min, a relatively prolonged rinsing step of 5 min at a flow rate of 0.5 ml/min was introduced to eliminate some memory effect observed in the initial set-up of the analytical method. This rinsing step was followed by the column-conditioning step with the initial solvent composition (98/2 solvent A/solvent B) at a flow-rate of 0.4 ml/min (2.4 min) and 0.3 ml/min (2.5 min).

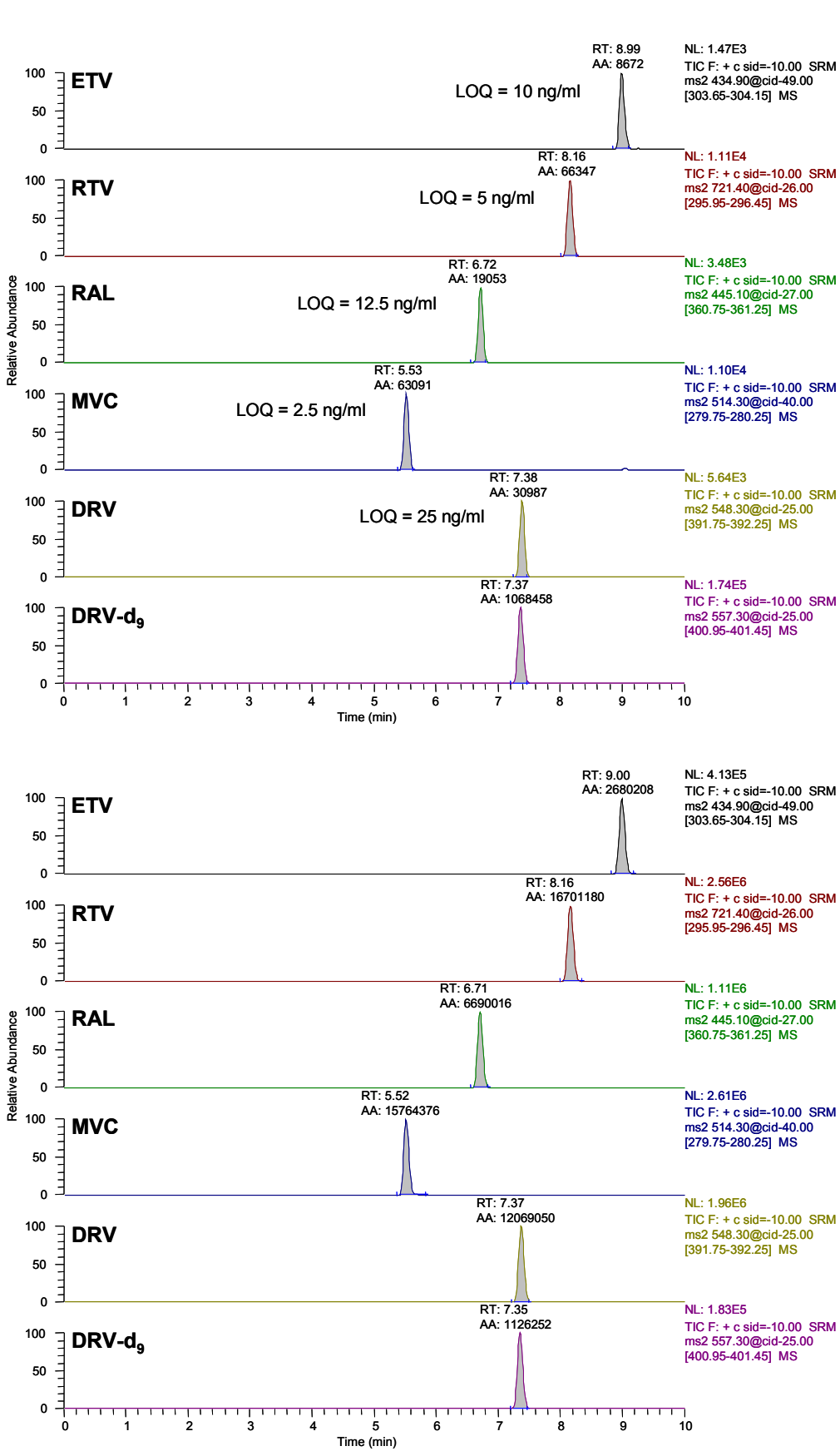


Figure 2: Chromatograms of (a) the lowest calibration sample (LOQ) and (b) the highest quality control sample containing DRV, ETV, MVC, RAL and RTV at concentrations reported in Table 1 (DRV-d_9 (I.S.) at 1000 ng/ml).

Figure 3 shows the signals at all selected *m/z* transitions when a single solution containing all antiretroviral drugs and I.S. was continuously infused post-column directly into the MS/MS detector during the chromatographic analysis of six different blank plasma extracts. The signals at the *m/z* transition showed a remarkably similar pattern, with all traces being essentially superimposable. Even though no marked matrix effect (no drifts or shifts of the signals) was observed at the respective retention time of the antiretroviral drugs and I.S. peaks (shown in the chromatographic profile) in this perfusion experiment, some matrix effects were however found as reported in the experiments below (see § 2.1.4.5).

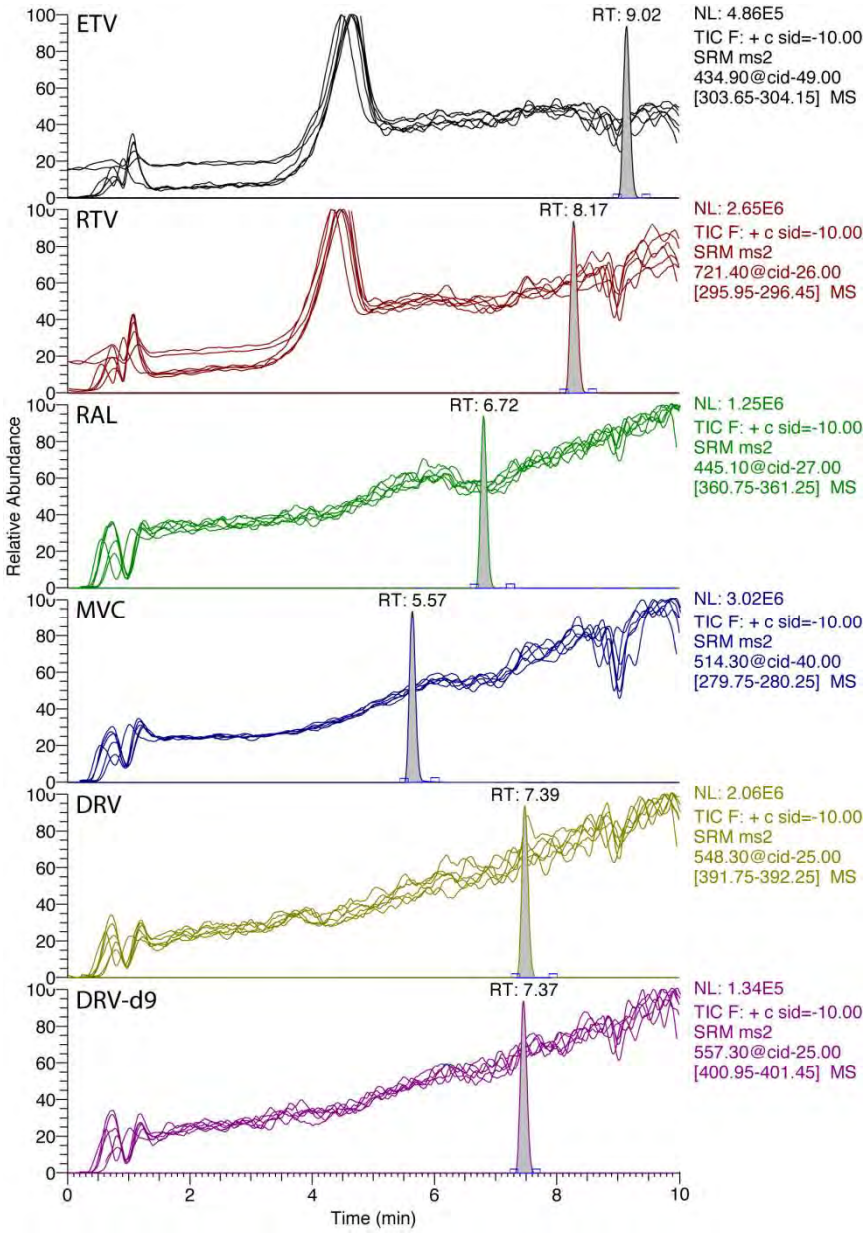


Figure 3: Chromatogram of 6 blank plasma extracts with post-column infusion of a solution containing DRV, ETV, MVC, RAL, RTV and DRV-d_9 at 100 ng/ml. Chromatogram of a calibration sample is also shown.

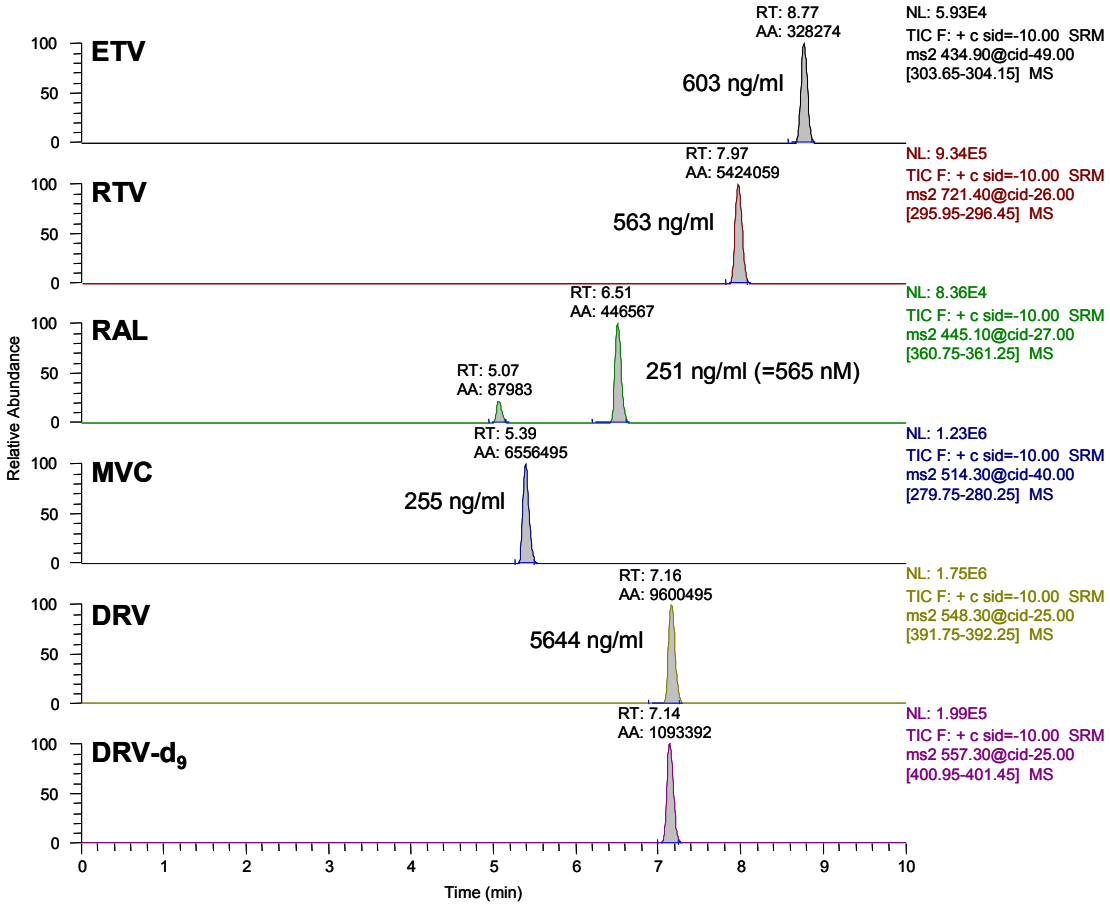
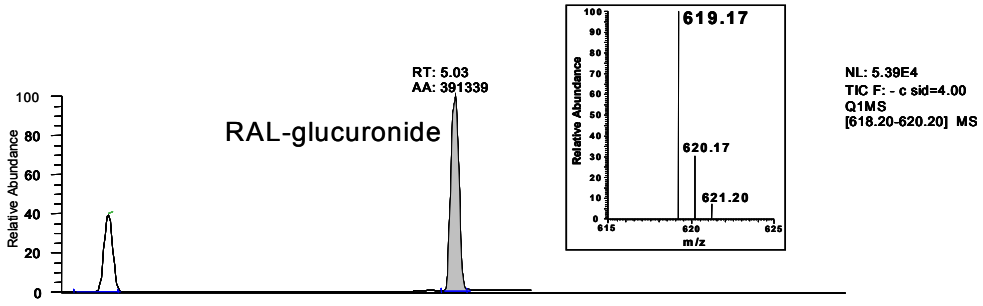


Figure 4: Chromatogram of a plasma sample from a patient receiving DRV, ETV, MVC, RAL and RTV. The blood sample was taken 1.5 h after drug intake (details in the text).

The chromatograms in Figure 4 were obtained from a patient under DRV (600mg BID), ETV (200mg BID), MVC (150mg BID), RAL (400mg BID) and RTV (100mg BID). The blood sample was taken 1.5 h after last drug intake. The plasma levels measured were 5644 ng/ml, 603 ng/ml, 255 ng/ml, 251 ng/ml (= 565 nM) and 563 ng/ml for DRV, ETV, MVC, RAL and RTV, respectively. Interestingly, the *m/z* transition selected for RAL showed in patients an additional peak at 5.07 min, eluted before RAL, which was hypothesized to be due to the in-source dissociation of raltegravir-glucuronide (RAL-gln), the only reported metabolite so far for RAL (18). This peak identification was confirmed in a separate sample analysis by single ion monitoring at $[M-H]^-$ = 619, corresponding to the molecular weight of RAL-gln (MW 620), using the negative mode and a lower collision induced dissociation (CID) energy (= 4 V). This is shown in the Figure 5 on the chromatographic profile A of a sample taken 1.5 h after RAL intake from a patient under 400mg BID. The chromatographic profile B in Figure 5 shows the standard analysis of the same sample in the positive mode, using the SRM *m/z* transition 445.1 → 361.0

selected for RAL, confirming that the early peak co-elutes with RAL-gln (upper profile A). The intensity of the signal for RAL-gln in this patient was found to be approximately 8-fold higher than that measured for RAL. In fact, with the proposed method, large interindividual differences in the extent of RAL glucuronidation have been observed so far, with individual RAL-gln/RAL ratios varying up to a 130 fold-factor. Of importance, these interindividual differences are not due to the variability in the efficiency of RAL-gln in-source fragmentation as RAL-gln/RAL ratios were found not to be significantly influenced by plasma matrix alteration (see 2.1.4.6). Some individuals have repeatedly RAL-gln plasma peak signals more than 10-fold higher than those measured for RAL.

Profile A (SIM, negative mode)



Profile B (SRM, positive mode)

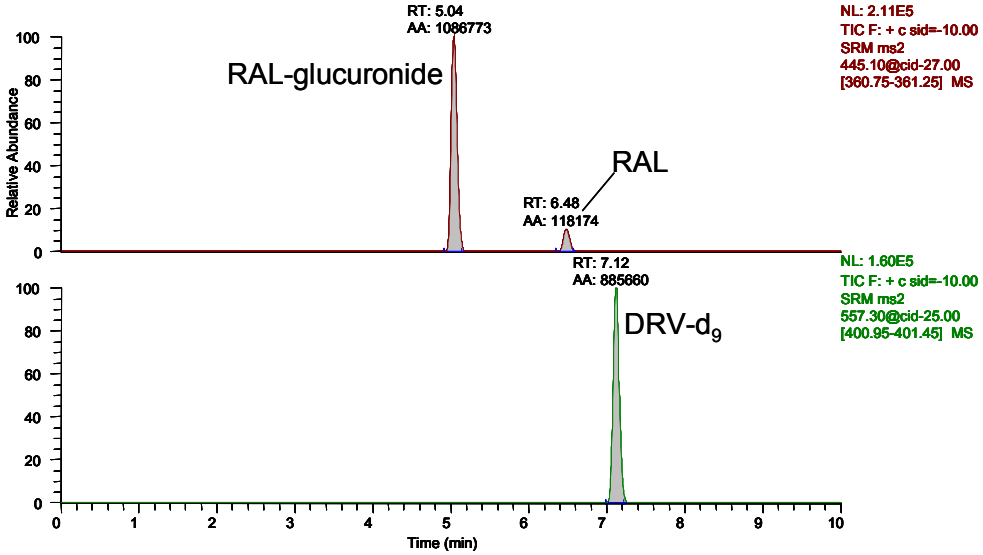


Figure 5: Chromatogram of a plasma sample taken 1.5 h after drug intake from a patient receiving RAL 400mg BID. This patient was also receiving EFV (600mg QD) and LPV/RTV (600/150mg BID).
Profile A: SIM in the negative mode at *m/z* $[M-H]^-$ = 619 selected for RAL-gln; *Offset*: MS spectra at the peak retention time for RAL-gln.
Profile B: standard SRM in the positive mode at *m/z* 445.1 → 361.0 selected for RAL; and transition for the I.S. DRV-d_9

Those data, although preliminary, departs strikingly from the only study on RAL metabolism published so far in healthy volunteers after a single raltegravir dose, which reports that RAL-gln accounts for 30% of total RAL in plasma (18). Whether the RAL-gln/RAL ratio constitutes a valid, clinically useful, marker of raltegravir glucuronidation is currently being evaluated as part of a controlled drug-drug and drug-gene interaction trial.

2.1.4.2. Internal standard and calibration curve

Ideally, deuterated analogues or homologues of the antiretroviral drugs would be the first-choice standards but these were not available to us. Several chemical compounds, unlikely found in patients but sharing some structural/chemical similarities with antiretroviral drugs, were evaluated as potential internal standards. Finally, DRV-d_9 was selected because it was used for the assay of darunavir, and had a negligible memory effect and, as a deuterated labelled compound, it is not present in patients.

For all antiretroviral drugs, calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactory described by either $1/x$ or $1/x^2$ weighted quadratic regression, or quadratic log-log regression, of the peak-area ratio of antiretroviral drug to I.S., *versus* the concentrations of the respective antiretroviral drugs in each standard sample.

Over the considered concentration range, regression coefficient r^2 of the calibration curves were always greater than 0.99 with back-calculated calibration samples within ±15% (±20% at LLOQ).

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bag may not fully reflect the plasma matrix from HIV patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibration samples preparation would be impracticable and difficult to justify from an ethical point of view. For the sake of validation, the cross-validation has been performed between four series of the three levels of QC and two series of calibration samples analysed in duplicate (citrate *versus* EDTA). Regression analysis of the head-to-head comparison show that the anticoagulant does not influences significantly the results for raltegravir, darunavir, maraviroc and ritonavir ($p = 0.98$, 0.57, 0.97, and 0.97, respectively). For etravirine, a slightly significant ($p=0.04$) bias of -5.9% was observed for EDTA samples analysed with citrate calibration. However, even if this observation was not spurious (simply due to test repetition without Bonferroni's correction), CI 95% values for this bias (–0.3 to –11.3%)

are comprised within the ±15% allowance for analytical deviation and are unlikely to be of any notable clinical significance.

2.1.4.3. Precision and accuracy, LLOQ, LOD

Precision and accuracy determined with the QC samples are given in Table 3. The levels of control samples were selected to reflect the low, medium and high range of the calibration curves chosen to encompass the clinical range of concentrations found in patients' plasma. The mean intra-assay precision was similar over the entire concentration range and always less than 8.9 %. Overall, the mean inter-day precision was good, with CV within 5.1-9.8 %. The intra-assay and inter-assay deviations (bias) from the nominal concentrations of each analysed antiretroviral drug were comprised between –5.9 and +9.7 %, and –3.3 to +5.1 %, respectively.

The results of the determination of LLOQ and LOD of antiretroviral drugs in plasma are shown in Table 4. By analysing plasma samples spiked with decreasing concentrations, the lowest achievable LOD among the considered antiretroviral drugs was obtained for maraviroc at 125 pg/ml. The lowest LOQ was obtained for MVC at 0.5 ng/mL, corresponding to an amount of 5 pg of drug into the 10 μl-injection volume. Overall, in the concentration ranges chosen for the establishment of the calibration curves (i.e. 12.5 - 5'000 ng/ml, 2.5 - 1'000 ng/ml, 25 - 10'000 ng/ml, 10 - 4'000 ng/ml and 5 - 2'000 ng/ml, for RAL, MVC, DRV, ETV and RTV respectively), the precision and accuracy of the lowest calibration sample were, for each antiretroviral drug, comprised within the ± 20% limit recommended by the FDA (38), in line with the latest updates of the Washington and Arlington Conference reports (39).

Table 4: Limit of detection and lower limit of quantification of antiretroviral drugs

	LOD	LOQ		Accuracy at LOQ	Precision at LOQ
	(ng/ml)	(ng/ml)	(pg)	(bias%)	(CV%)
Darunavir	1.25	10.0	100	+0.02	4.1
Etravirine	2.5	10.0	100	–0.06	7.9
Maraviroc	0.125	0.5	5	–2.27	2.0
Raltegravir	1.25	7.8	78	–3.24	13.7
Ritonavir	1.0	1.0	10	–3.30	18.0

Of note, the chosen ranges of calibration were selected initially to cover the *clinically relevant* range of antiretroviral drug concentrations presumably present in the plasma samples collected during the medical visit generally taken at random time after the last

drug intake. Except in some cases of non compliance to treatment, plasma drug levels encountered so far have been all lying above the lowest calibration levels. During the course of the method validation however, it was observed *a posteriori* that the performance of our tandem MS/MS detector enables us to attain lower detection and quantification limits, well below the clinically relevant range of antiretroviral drugs concentrations established during the validation procedure. These LLOQ and LOD attained were found to differ depending of the drug considered, and in the perspective of this method validation, these results are reported in Table 4. However, for the sake of standardization and for the simplification of solution preparation, it was decided to keep the same *clinically relevant* range of calibration previously determined for all drugs, regardless of their LLOQ.

2.1.4.4. Stability of antiretroviral drugs

The stability of antiretroviral drugs in plasma QCs samples left at room temperature (RT) and at +4°C was ascertained up to 48 h. The variation over time of each drug levels at 24h and 48h (Table 5) was mostly comprised within the ± 15% of nominal concentrations (except for two values from ETV and RTV, where T24 exceed 15%) indicating that, taking into account the analytical variability, antiretroviral drugs can be considered generally stable.

Table 5 shows the variation of antiretroviral drug concentrations after one, two and three freeze-thaw cycles. For RAL, MVC, ETV and RTV, the variation was always less than 15%, indicating no significant loss of drug after up to three freeze-thaw cycles. For DRV, the variation after the third cycle at the highest concentrations tested slightly exceeded 15% but remained however below 20%.

Calibration and quality control samples wee prepared in batches, distributed and stored at –20°C in 4 ml polypropylene tubes for use up to one month in our routine antiretrovirals monitoring facility. No evidence of raltegravir, etravirine, darunavir and maraviroc decomposition was found during plasma samples storage in the freezer at -20°C for at least 6 weeks. In the same conditions, a mean 10% decrease in ritonavir levels was noticeable but this variation remains always less than the ± 15% allowance and should not affect to a clinically relevant extend ritonavir plasma levels determination.

Table 5: Stability of antiretroviral drugs in human plasma QC samples

Drug		Darunavir			Etravirine			Maraviroc		
Nominal concentration [ng/mL]		250	1250	7500	100	500	3000	25	125	750
Room temperature	24h	5.7	-6.6	7.0	2.2	-9.1	-9.3	3.3	2.2	0.3
	48h	-2.1	3.2	-1.3	-13.5	-5.4	-9.7	2.1	8.2	-4.2
+4°C	24h	6.8	5.2	2.5	-24.0	-4.6	-6.6	0.7	5.6	-12.2
	48h	-5.6	-9.0	-0.3	-11.5	-9.7	-0.2	-4.3	-7.4	-3.6
Freeze-thaw	cycle 1	-7.4	-4.7	-5.2	0.1	5.1	-0.5	-0.4	-2.8	-3.3
	cycle 2	-11.6	-7.2	-2.2	-14.9	3.6	1.5	-6.6	-6.7	-5.4
	cycle 3	-15.4	-9.3	5.8	-1.5	0.1	6.7	0.1	-4.0	2.3

Drug		Raltegravir			Ritonavir		
Nominal concentration [ng/mL]		125	625	3750	50	250	1500
Room temperature	24h	2.9	4.9	0.7	-2.7	1.6	4.9
	48h	-4.8	6.3	3.3	-5.6	1.5	-1.5
+4°C	24h	5.0	7.9	-2.7	-7.4	5.6	-19.2
	48h	-14.4	-14.5	10.5	-14.2	-9.1	14.9
Freeze-thaw	cycle 1	8.0	-0.8	-6.8	-1.9	-4.7	2.8
	cycle 2	3.2	-4.7	-4.9	-1.7	-6.0	-8.5
	cycle 3	5.9	0.1	0.5	5.5	-3.3	2.4

Of note, calibration samples subjected to the thermal viro-inactivation procedure (60 min at 60°C in a water-bath) are not stable thereafter upon storage at –20°C, indicating that calibration samples must be subjected to thermization procedure only on the day of analysis.

2.1.4.5. Matrix effect and recovery

Matrix effect was examined by the simultaneous post-column infusion of antiretroviral drugs and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma extracts from blood donors (see § 2.1.4.1.). Co-eluting matrix components may nevertheless reduce or enhance the ion intensity of analytes, possibly affecting the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all standard calibrations and quality control samples have been prepared by spiking drugs in human plasma.

Table 6: Matrix effect, extraction recovery, analysis recovery and process efficiency of antiretroviral drugs.

Component	Nominal conc. [ng/ml]	Mean peak area			Mean peak area ratio		ME [%]	CV [%]	ext RE [%]	CV [%]	Analysis RE [%]	Mean	CV [%]	PE [%]	CV [%]
		A (n = 3)	B (n = 6)	C (n = 6)	B2	C2	B/A		C/B		C2/B2			C/A	
Darunavir	250	333517	344809	323650	0.301	0.298	103.4	9.6	93.9	12.3	99.1	101.1	2.7	97.0	5.9
	1250	1574728	1680867	1658152	1.467	1.528	106.7	1.5	98.6	2.9	104.2			105.3	3.6
	7500	9207611	10457052	9916646	9.124	9.136	113.6	3.2	94.8	6.7	100.1			107.7	7.4
Etravirine	100	139647	116067	111041	0.101	0.102	83.1	10.0	95.7	11.9	101.0	100.5	4.1	79.5	4.4
	500	591132	583261	575618	0.509	0.530	98.7	4.8	98.7	4.4	104.2			97.4	5.9
	3000	2992636	3195373	2909228	2.788	2.680	106.8	3.5	91.0	7.2	96.1			97.2	6.0
Maraviroc	25	730295	768013	761102	0.670	0.701	105.2	4.4	99.1	5.0	104.6	106.1	1.2	104.2	3.2
	125	3492992	3618014	3662107	3.157	3.374	103.6	4.1	101.2	3.3	106.9			104.8	1.7
	750	17326261	18813088	19007243	16.414	17.512	108.6	2.9	101.0	4.5	106.7			109.7	3.8
Raltegravir	125	246764	236088	237709	0.206	0.219	95.7	8.1	100.7	12.0	106.3	104.2	2.7	96.3	4.7
	625	1141426	1211486	1206641	1.057	1.112	106.1	3.9	99.6	5.8	105.2			105.7	3.2
	3750	6419404	7187229	6882970	6.271	6.341	112.0	3.2	95.8	7.1	101.1			107.2	7.9
Ritonavir	50	597795	767365	738231	0.670	0.680	128.4	2.9	96.2	8.4	101.6	100.8	0.7	123.5	6.3
	250	2879597	3613948	3429056	3.153	3.159	125.5	7.8	94.9	3.0	100.2			119.1	5.7
	1500	15390132	19482061	18558090	16.998	17.098	126.6	3.4	95.3	6.0	100.6			120.6	7.2
Darunavir-d_9 (IS)	1000	1077729	1146132	1085390			106.3	3.5	94.7	5.0				100.7	4.0

A = Peak area of standard solutions without matrix and without extraction; B = Peak area of analytes spiked after extraction; C = Peak area of analytes spiked before extraction; B2 = Ratio of the peak area of the analyte and the I.S. spiked after extraction; C2 = Ratio of the peak area of the analyte and the I.S. spiked before extraction; ME = Matrix effect expressed as the ratio of the mean peak area of the analytes spiked after the extraction (B) to the mean peak area of the same standard solution without matrix (A) multiplied by 100; ext RE = Extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (C) to the mean peak area of the analytes spiked after extraction (B) multiplied by 100; Analysis RE = Analysis recovery calculated as the ratio of the mean peak area ratio of the analytes spiked before extraction (C2) to the mean peak area ratio of the analytes spiked after extraction (B2) multiplied by 100; PE = Process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (C) to the mean area of the same analyte standard (A) multiplied by 100.

The assessment of the *matrix effect* (Table 6) was quantified as the peak-area response of analytes added to blank plasma extracts (i.e. B, drugs added *after* extraction), expressed as the percentage of the response of standard solution of drugs directly injected onto the column (A) (ratio B/A, in Table 6). A value above or below 100% for the matrix effect indicates an ionization enhancement or suppression, respectively. The results indicate that co-eluting plasma matrix components do not appear to affect significantly the ionisation of RAL, MVC, ETV and DRV (mean ratio B/A =104%). In the spiking experiments, an ionization enhancement of 27% was noticed for RTV but this effect was found remarkably stable not only at the three concentrations studied (128.4 ± 3.8%, 125.5 ± 9.8%, 126.6 ± 4.3%, for low, medium and high concentration, respectively) but also amongst the 6 different plasma matrices (variability < 7.8%). Conversely, in the perfusion experiments, a small drop in the signal could be noticed at the retention time of ETV (Figure 3). However, in the spiking experiments (Table 6) this showed to slightly affect the ME for the lower QC sample only (83%), with an acceptable reproducibility (CV 10%).

The *analytical recovery* was calculated in a similar way, but considering drugs response/I.S. ratio instead, before (C2) and after (B2) the extraction procedure (ratio C2/B2, in Table 6). Taking into account the I.S. response enables to correct for the occurrence of variation over time in the MS/MS spectrometer performance and injection volume. As indicated in Table 6, the extraction recovery for the I.S. was essentially 95%. The *analytical recoveries* achieved were always > 96% for all antiretroviral drugs.

The *process efficiency* (i.e. overall recovery) given in Table 6 (column *PE*) was obtained as the peak-area response of analytes spiked into plasma samples *before* the extraction procedure (C) – such as calibration and control samples – expressed as the percentage of the peak area of pure drug standard solution (A) directly injected into the column. This *process efficiency* takes into account the analytical recovery and the matrix effect: for example, DRV has a mean matrix-mediated ionization enhancement close to 108% (Table 6, column *ME*), which combined with a mean extraction yield of 96% (Table 6, column *ext RE*) gives an process efficiency around 103%. Overall, these results indicate that even though no apparent matrix effect was observed in the infusion experiment (Figure 3), matrix components do influence to some extent the overall process efficiency. This was especially noticeable for RTV at all QCs concentrations, and to a lesser extent ETV at the low QC level, requiring therefore the preparation of calibration and control samples in plasma matrix reflecting at best the composition of the samples

to be analysed. Most importantly, it is not so much the matrix effect *per se* that must be reduced than its variability.

As shown in Table 6, the variability of the matrix effect of 6 different plasma matrices never exceeded 10.0%, demonstrating indeed that the proposed extraction procedure is able, if not to eliminate, at least to normalize and standardize the matrix effect.

2.1.4.6. Influence of plasma matrix variability on raltegravir-glucuronide/ raltegravir ratios values

In patients samples containing high amounts of RAL-gln, RAL-gln/RAL ratios values were not affected by sample alteration upon dilution 1:2 with 6 different sources of plasma. In patient samples with only small amounts of RAL-gln, some differences in RAL-gln/RAL ratios were noticed upon plasma matrix alteration but those differences were comparatively small, and of no clinical significance in the context of studies on patient's raltegravir glucuronidation rate. Overall, this indicates that any variability in plasma matrix composition does not affect, or only slightly RAL-gln/RAL ratios values. Importantly, the extremely high RAL-gln/RAL ratios observed in some individuals are thus not due to matrix effects that would have influenced the extent of RAL-gln in-source dissociation.

2.1.4.7. Dilution effect

After the 5-fold dilution of the spiked plasma with antiretroviral drugs at a concentration exceeding by two-fold the high calibration level, the mean deviation (bias) from the expected concentrations was +1.0%, –0.3%, –2.2%, +4.0% and –6.6% for DRV, ETV, MVC, RAL and RTV, respectively. This indicates that plasma samples containing antiretroviral drugs above the high level of calibration can be adequately diluted with blank plasma prior to the LC-MS/MS analysis.

2.1.4.8. Clinical applications

This analytical method has been shown reliable and sensitive for monitoring plasma concentrations of RAL, MVC, DRV, ETV and RTV in patients as part of our routine TDM service and for clinical research projects done within the frame of the Swiss HIV Cohort Study.

Figure 6 shows the plasma levels, plotted against time after drug intake, measured for DRV, ETV, RAL and MVC, given either separately, or in combination (see introduction), or all four drugs altogether, with an optimized background regimen. Available data show

a very high inter-individual variability in RAL and ETV plasma levels in this experienced patient' population: these two drugs have therefore been prioritized for further investigations on the genetic and environmental factors that potentially influence their disposition. These studies are currently underway.

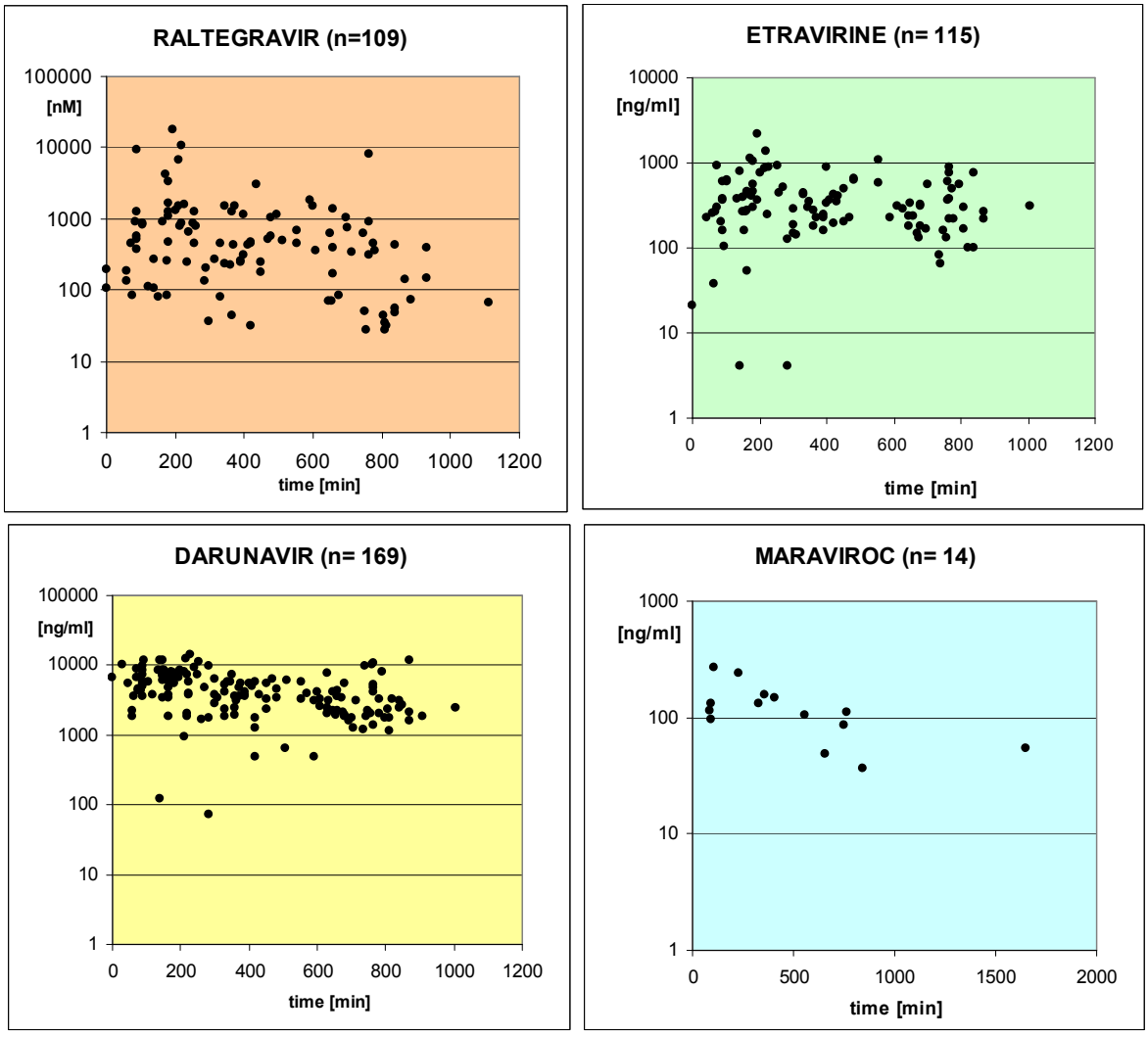


Figure 6: Plasma levels, plotted against time after drug intake of the latest four antiretroviral drugs

2.1.5. Conclusion

The reported and validated LC-MS/MS method provides a robust procedure for determining four novel antiretroviral drug concentrations in plasma, enabling their monitoring and a better understanding of their efficacy, toxicity and potential for drug interactions. The high sensitivity and specificity attained with LC-MS/MS enable the quantification of as little as 5 to 100 pg on-column, depending on the drug (Table 4), in an aliquot as low as 100 μl volume.

This is, to the best of our knowledge, the first validation report for a LC-MS/MS method for the simultaneous assay of antiretroviral agents targeted to four different steps of HIV replication, using the convenience of a single-step extraction and an elution gradient program allowing their quantification in the same analytical run.

The advent of novel drugs from current and new antiretroviral classes increases the complexity of use of antiretroviral drugs for effectively maintaining patients' health through the dual goal of long term virological suppression with minimal toxicity. All the new agents have the potential for significant interactions with other antiretroviral medications, adding to the challenge for clinicians to devise long-term effective antiretroviral regimen combinations, while still having to deal with the complexity of previous PI- and NNRTI-based regimens and their associated drug interactions. With doubts hanging over the long-term effect of chronic CCR5 inhibition and the limited experience so far with the first integrase inhibitor raltegravir, a continuing monitoring seems essential for CCR5 antagonists and HIV integrase inhibitors. It is however too early to appreciate whether measuring these new antiretroviral drugs in plasma will add clinically useful information to the current TDM performed for PIs and NNRTIs in the follow-up of HIV patients.

This analytical method is suitable for monitoring new antiretroviral drugs, for suggesting lack of short-term compliance and for helping in the identification of drug interactions in new regimens modifying the systemic disposition of drugs to an extent likely to impair virologic response.

Our method suitably answers the demands of clinicians for monitoring novel drugs administered in combination, often as salvage therapy, to heavily pre-treated patients, in whom exposure, tolerance and adherence assessments are critical issues. Further studies will determine its contribution to risk minimization and to therapy optimization.

2.1.6. References

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2.2. Free drug concentration

In plasma, drugs are present in two forms: bound to plasma proteins (mostly human serum albumin, HSA, and α-1-acid glycoprotein, AAG) and unbound or free (Figure 1). The free drug is the only form likely to diffuse into tissues and to penetrate into cells to exert antiretroviral activity. Whereas PIs are highly bound to plasma proteins, mostly to AAG, NNRTIs bind predominantly to HSA.

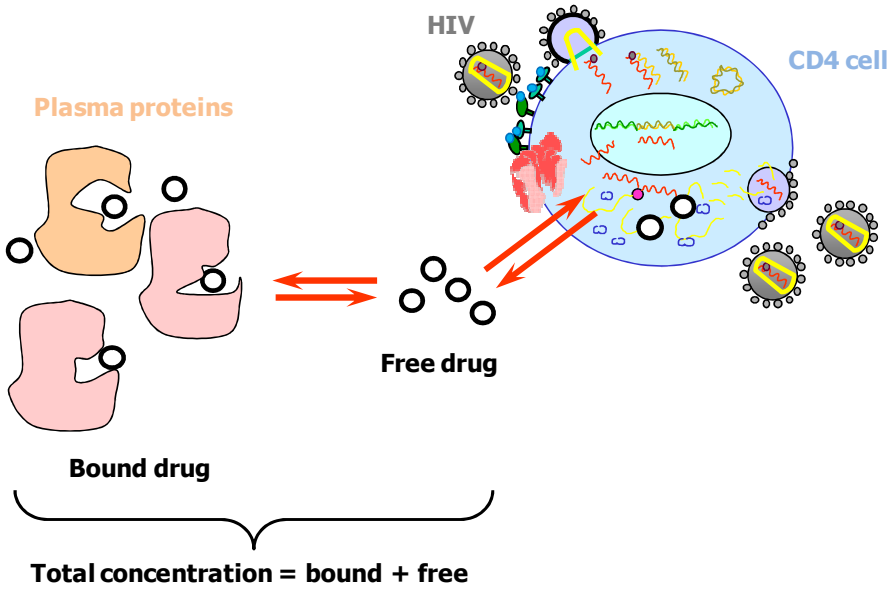

Figure 1: Schematic representation of drug protein binding and penetration into target cell.

Currently, total (bound + free) plasma concentrations (C_{tot}) of ARVs are measured in patient plasma for Therapeutic Drug Monitoring (see Chapter 3), presuming that C_{tot} are a valid surrogate for free concentrations (C_{free}), the only form exerting antiviral activity. As diseases or infections, as well as pregnancy, can significantly alter protein binding, thus modifying free fraction, determination of free instead of total concentrations may provide a more accurate indicator of "effective" drug exposure and contribute to better ARV therapy individualisation.

To date, several methods to measure C_{free} have been published (see below), but most of them are time-consuming, necessitate complex instruments not suitable for routine use, or are biased by several problems (adsorption onto devices, dilutional shifts in equilibrium,…). Thus, a convenient and accurate method for the measurement of C_{free} of antiretroviral drugs has been developed and is presented here.

[See also related Appendix 2.2]

DETERMINATION OF UNBOUND ANTIRETROVIRAL DRUGS CONCENTRATIONS BY A MODIFIED ULTRAFILTRATION METHOD REVEALS HIGH VARIABILITY IN THE FREE FRACTION

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pdf version: Appendix 2.3.

2.2.1. Abstract

Total plasma concentrations are used for Therapeutic Drug Monitoring of antiretroviral drugs, whereas antiviral activity is expected to depend on unbound concentrations. The determination of free (unbound) concentrations by ultrafiltration may be flawed by the irreversible adsorption of many drugs onto the membrane filters and plastic components of the device.

The authors describe a modified ultrafiltration method enabling the accurate measurement of unbound concentrations of 10 antiretroviral drugs by LC-MS/MS, which circumvents the problem of the loss by adsorption in the early ultrafiltration fractions. The method was applied to assess the variability of free fractions of antiretroviral drugs during routine TDM in 144 HIV patients.

In *in vitro* experiments, ultrafiltrate collected in four fractions (0-8, 8-16, 16-24 and 24-30 minutes) gave much lower and more variable free drug concentrations in the first ultrafiltrate fraction than in the last three fractions for lopinavir, nelfinavir, saquinavir, tipranavir and efavirenz. In the last two fractions, free concentrations remained constant, indicating a saturable adsorption. The adsorption was modest for indinavir, amprenavir and ritonavir, and unnoticeable for atazanavir and nevirapine. Free fraction values obtained with this modified ultrafiltration method reveals substantial inter-individual variability, suggesting that monitoring unbound antiretroviral drug concentrations may increase its clinical utility, especially for lopinavir, saquinavir and efavirenz

2.2.2. Introduction

Drugs are present in two forms in plasma: bound to plasma proteins (mostly albumin and α-1-acid glycoprotein) and unbound (free). The unbound drug is usually considered the only form diffusing into tissues and penetrating into cells to exert its activity. Wide differences exist in the extent of binding of antiretroviral drugs (ARVs). HIV protease inhibitors (PIs) are mostly lipophilic weak basic molecules highly bound to plasma proteins (> 85% except indinavir, 60%), mainly to α-1-acid glycoprotein (AAG). The non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine, weakly acidic, bind predominantly to albumin (1) (> 99% and 60%, respectively).

Total (bound + free) concentrations are currently determined for Therapeutic Drug Monitoring (TDM) of ARVs (2, 3). A small change in the extent of protein binding of highly bound drugs results in a dramatic effect on the free fraction (4, 5). Diseases or infections can significantly alter the binding of drugs (4-7). Pregnancy also affects

protein binding (4, 8-13). Recently, phenotypic variants of AAG have been found to influence the clearance and distribution of some PIs (14). For NNRTIs, total plasma concentrations correlate only modestly (efavirenz) or not at all (nevirapine) with cellular concentrations measured in peripheral blood mononuclear cells (15), thus prompting investigations to identify whether variability in protein binding could explain this discrepancy.

Information on free drug concentrations and corresponding free fractions (free/total concentration ratio) could clarify the exposure to unbound ARVs induced by physiological, genetic, pathophysiological conditions or pharmaceutical interactions, and improve the contribution of TDM to treatment adjustment. Indeed, changes in free fraction can influence the interpretation of total drug measurement. For drugs of *low* extraction, a change in protein concentrations or binding affinity (albumin and/or α-1-acid glycoprotein) alters total plasma concentrations while free drug concentrations remain mostly unchanged (16). Conversely, changes in protein binding for drugs of *high* extraction are not expected to alter *total* drug concentrations while free drug concentrations are affected. In both cases, altered free fractions impact on total concentration-effect relationships and may compromise the correct interpretation of TDM results. Therefore, unbound drug concentration determination may provide a more accurate indicator of "effective" drug exposure and contribute to a better individualization of drug dosage regimens (1, 4, 17, 18).

Equilibrium dialysis and ultrafiltration are the methods most frequently used for free drug concentration determination (4, 5). Although equilibrium dialysis is considered the reference method for determining drug-protein binding, its results may still be biased by dilutional shifts in equilibrium caused by diffusion of free fraction into the dialysate, osmotic dilution of retentate, incomplete attainment of dialysis equilibrium, binding to buffer ions and non-specific drug adsorption onto the dialysis apparatus. But the main drawback of equilibrium dialysis is the tedious, time-consuming procedure, not suitable for routine use in clinical laboratories. By contrast, ultrafiltration is relatively quick and easily implemented for large numbers of samples. However, as for dialysis equilibrium, non specific binding to membranes and plastic components of ultrafiltration devices remains an issue potentially leading to underestimation of the unbound concentration (4, 5, 19-21). While ultrafiltration has been proposed for the determination of free concentrations of ARVs, irreversible adsorption onto device components was reported to underestimate the free fraction of nelfinavir and lopinavir by as much as 25% (17) and 50% (22), respectively. On the other hand, adsorption seems negligible for amprenavir

(23) and indinavir (24). For saquinavir and tipranavir, the adsorption issue has not yet been addressed.

Such limitations probably explain why information on free concentrations of ARVs remains scarce. The few published investigations report small numbers of subjects receiving PIs (17, 22-37) or NNRTIs (38, 39) followed over one dosing interval. Free and total concentrations were found roughly correlated, but with substantial interindividual variability in free fraction.

We aimed at developing a method for the convenient and accurate measurement of unbound concentrations of ARVs (amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), tipranavir (TPV), efavirenz (EFV) and nevirapine (NVP)) using the method of ultrafiltration, while circumventing the loss by adsorption. The method was applied to the assessment of inter-individual variability in antiretroviral drug free fractions and to their correlation with albumin and AAG levels in HIV patients undergoing routine TDM.

2.2.3. Material and methods

2.2.3.1. Chemicals and reagents

ATV was kindly provided by Bristol-Myers Squibb (Baar, Switzerland), APV by GlaxoSmithKline (Stevenhage, UK), EFV and IDV by Merck Sharp & Dohme-Chibret (Glattburg, Switzerland), LPV and RTV by Abbott (Abbott Park, Illinois, USA), NFV and SQV by Roche (Basel, Switzerland), NVP and TPV by Boehringer Ingelheim (Ridgefield, CT, USA). The research compound A-86093 given by Abbott was used as Internal Standard (I.S.) for the assay. Of note, deuterated analogues of most ARVs have been made available only recently (i.e. d4-efavirenz, d8-lopinavir, d5-atazanavir, d5-nevirapine) (LGC Promochem, Molsheim, France) and should certainly be considered as the optimal I.S. at present.

Solvents (methanol (MeOH), acetonitrile (MeCN) LiChrosolv® grade, 100% formic acid) were purchased from Fluka (Buchs, Switzerland) and ammonium acetate from Merck. Ultrapure water was obtained through a Milli-Q® UF-Plus apparatus (Millipore Corporation, Burlington, MA, USA).

2.2.3.2. Equipment

The HPLC system involved a Rheos 2200 binary pump (Flux Instruments, Basel, Switzerland) equipped with an online degasser and a temperature-controlled 324 vial

autosampler maintained at +10°C (CTC Analytics AG, Zwingen, Switzerland). The chromatographic system was coupled to a triple stage quadrupole mass spectrometer (TSQ Quantum Ion Max) from Thermo Electron Corporation (Waltham, MA, USA) equipped with an electrospray ionisation (ESI) interface and operated with the Xcalibur 1.1 software (Thermo Electron Corporation, San Jose, CA, USA).

2.2.3.3. Liquid chromatography-mass spectrometry chromatographic conditions

Chromatographic separations were performed on an Atlantis® dC18 column (2.1 x 50 mm, 3 μm) (Waters, Milford, MA, USA) in a thermostated column-heater at 25°C (Croco-Cil®, Cluteau Info Labo).

Solution A consisted of 2 mM ammonium acetate containing 0.1% formic acid. Solution B was MeCN with 0.1% formic acid. Solution C consisted of MeCN with 1% formic acid. The mobile phase was delivered at 0.3 mL/min according to the following gradient elution program: 0-1 min, 2% solution B → 30% B at 3.5 min, → 100% B at 10 min. The washing/re-equilibration step included an intensive rinsing (10-14 min with 100% of C at 0.5 mL/min, → 100% B at 15 min at 0.5 mL/min) followed by the initial solvent composition (2% solution B : 15.1-18 min at 0.4 mL/min, and 18-20 min at 0.3 mL/min). For EFV analysis, solutions A and B were prepared without formic acid, enabling an enhanced sensitivity. The chromatographic separation of EFV and I.S. was obtained by a step-wise elution 0-1 min, 2% solution B → 45% B at 2 min, → 45% B at 16.4 min, followed by rinsing and re-equilibration steps up to 21 min. Quantification of ARVs was performed using selected reaction monitoring (SRM) in the positive mode for all ARVs, EFV only being monitored in the negative mode. We used selected transitions and collision energies reported previously for PIs and NNRTIs (40), with some minor modifications. The *m/z* transition, collision energy (V) and tube lens used for TPV were 603.0 → 585.0, 25 and 77, respectively.

2.2.3.4. Plasma and ultrafiltrate stock matrix preparation

Outdated blood transfusion bags were aliquoted in 50mL-aliquots into Falcon® tubes and centrifuged without delay at 1850g (3000 rpm) for 10 min at +4°C (Beckmann Centrifuge, Model J6B). The blank plasma was used directly for the *in vitro* experiments or, alternately, transferred into an Amicon Centricon® Plus-20 Filter System (cutoff 30 kDa; Millipore Corporation, Bedford, MA, USA) and submitted to ultrafiltration (1850g, +4°C, 30 min) to obtain the blank ultrafiltrate pool for the preparation of calibration and

quality control samples. Aliquots (100 μL) of ultrafiltrate were distributed in Eppendorf vials and stored at -20°C until use.

2.2.3.5. Ultrafiltrate calibration and quality control samples

Stock solutions at 1 mg/mL in MeOH for APV, ATV, EFV, NFV, RTV, SQV, at 1 mg/mL in MeOH/H_2O (50:50) for IDV and NVP, at 2 mg/mL in MeOH for LPV, and at 5 mg/mL in MeOH for TPV, were diluted with MeOH for the preparation of working solutions at concentrations of 2-10'000 ng/mL. On the day of analysis, a 100 μL-volume of each working solution was added to 100 μL ultrafiltrate, to reach concentrations of 1, 5, 10, 50, 100, 500 and 1'000 ng/mL for calibrators (5000 ng/mL for IDV and NVP), and concentrations of 2.5, 25, and 200 ng/mL for quality controls.

2.2.3.6. Total calibration and quality control samples

Blank plasma samples were spiked with stock solutions appropriately diluted to reach the clinically relevant total plasma concentrations for calibration: 50-10'000 ng/mL for APV, ATV, EFV, IDV, NFV, NVP, RTV, SQV; 100-20'000 ng/mL for LPV; 1'875-75'000 ng/mL for TPV, and for quality controls: 750, 3'000 and 9'000 ng/mL for APV, ATV, EFV, IDV, NFV, NVP, RTV, SQV; 1'500, 6'000 and 18'000 ng/mL for LPV; 5'625, 22'500 and 67'500 ng/mL for TPV (total added volume MeOH $\leq$ 10 % of the biological sample volume, in accordance with the recommendations on bioanalytical method validation (41-43).

2.2.3.7. Calibration curves

The calibration curve was measured with a series of calibration samples analysed in duplicate at the beginning and at the end of the run.

Quantitative analysis of PIs and NNRTIs in ultrafiltrate and plasma were performed using the internal standard (I.S.) method.

2.2.3.8. Determination of free drug concentrations

In vitro experiments with test samples

Prior to use, the ultrafiltration Amicon Centrifree® Filter Systems (cutoff 30 kDa; Millipore Corporation, Bedford, MA, USA) were conditioned with 500μL of ultrapure water submitted to ultrafiltration (2000g, 30 min, 26°C) in a fixed-edge, temperature-controlled centrifuge (Avanti® J-30I High Performance Centrifuge System, Beckmann,

USA). In the *in vitro* experiments, blank plasma aliquots (500μL) spiked with ARVs at two clinically relevant concentrations (750 and 2'500 ng/mL for APV, ATV, EFV, IDV, LPV, NFV, NVP, RTV, SQV, and 10'000 and 60'000 ng/mL for TPV) were placed in pre-washed Centrifree® ultrafiltration tubes, and subjected to ultrafiltration at 2000g (3650 rpm). The ultrafiltrate was collected in four fractions (0-8, 8-16, 16-24 and 24-30 minutes) in pre-weighed plastic cups. Each ultrafiltrate fraction was weighed accurately and diluted 1:1 w/v with MeOH without delay. After the addition of 20μL of I.S. solution (A-86093, 25 ng/mL in MeOH/H_2O 50:50) to 100μL-aliquot of each ultrafiltrate fraction, the resulting samples were introduced into 500μL HPLC microvials. A 10μL-volume was used for the LC-MS/MS analysis.

For overall comparison, free drug concentrations were measured in ultrafiltrate from spiked blank plasma samples collected (a) as a single (0-30 min) collection, according to the recommendations of the manufacturer, or (b) the 15-30 min-only ultrafiltrate collection.

2.2.3.9. Application to clinical samples

The method described was applied to the measurement of free drug concentrations of antiretrovirals in patients for whom monitoring of total plasma concentrations was requested as part of their medical follow-up.

Clinical samples were collected at random time after drug intake and processed in our laboratory as part of our routine TDM Service. Briefly, blood samples (5 mL, EDTA) were collected as previously described and centrifuged at 1850 g (3000 rpm) for 10 min at +4° C (Beckmann Centrifuge, Model J6B). The plasma was divided into two aliquots, one for total plasma concentration monitoring and the other for free concentration determination. The first aliquot was heated at 60°C for 60 min in a thermostated water bath (Memmert® WB 7, Schwabach, Germany) for viral inactivation (44-47). The stability of ARVs has been demonstrated under those conditions (48-51). Total concentrations were determined by LC-MS/MS after protein precipitation with acetonitrile using an adaptation of our previously reported method (40). In brief, a 100 μL-aliquot of patient plasma was mixed with 100 μL of I.S. solution (5 μg/mL for all ARVs, except for EFV, 80 ng/mL) and 600 μL of acetonitrile, vortexed and sonicated for 10 min (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was again vortex-mixed and finally centrifuged at +4°C for 10 min at 20'000g (14000 rpm) on a Hettich® Benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). An aliquot of the supernatant was diluted 1/75 (1/6 for EFV) with 20 mM ammonium acetate/MeOH

(50:50). A 10μL-volume was injected into the LC-MS/MS instrument for the determination of total drug concentrations. The laboratory participates in an international external quality assurance program for the analysis of total concentrations of antiretroviral drugs (KKGT, *Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie*, Association for Quality Assessment in TDM and clinical Toxicology, The Hague, The Netherlands).

The viral inactivation process was omitted in the second plasma aliquot to prevent alteration of protein binding. Free drug concentration was measured in duplicate in the second aliquot as follow: 500μL of plasma was submitted to ultrafiltration and collected as two fractions (0-15 and 15-30 minutes) in pre-weighed plastic cups. The 15-30 min ultrafiltrate collections were diluted 1:1 w/v with MeOH without delay. To 100μL-aliquots of each ultrafiltrate collection 20 μL of I.S. solution (A-86093, 25 ng/mL in MeOH/H_2O 50:50) was added and the solution was analyzed by LC-MS/MS. The volume injected into the LC-MS/MS was 10 μL. The free fraction was calculated in each sample as the ratio of the free and the total concentration, expressed as a percentage.

Because HIV patients were treated using a combined regimen with, for most, more than one drug subject to TDM (including RTV as pharmacokinetic enhancer), the number of drug measurements exceeds the number of samples.

The study was purely observational and no dose recommendations were made on the results of free drug concentrations.

2.2.3.10. Plasma albumin and α-1-acid glycoprotein concentrations

The albumin and AAG concentrations were measured using commercially available assays from Roche Diagnostics, with colorimetric and immunoturbidimetric methods, respectively, carried out on a Roche Cobas Integra 400 (Roche Diagnostics, Rotkreuz, Switzerland).

2.2.3.11. Data analysis

The correlation between total (C_{tot}) and free (C_{free}) concentrations and between free fractions and sampling times was assessed by standard linear regression analysis. The influence of AAG and albumin levels on total and free concentrations was examined by covariance analysis (ancova) by incorporating AAG and albumin levels in the equation $C_{tot} = C_{free} + C_{free}*[albumin] + C_{free}*[AAG]$, and examining the significance of their coefficient and their impact on the correlation coefficient.

Statistical significance was assigned at a p-value less than 0.05.

2.2.4. Results

2.2.4.1. Liquid chromatography-tandem mass spectrometry analysis

Matrix effect of ultrafiltrate

A potential matrix effect was examined by the simultaneous post-column infusion of PIs/NNRTIs and I.S. into the MS/MS detector during the chromatographic analysis of 6 different batches of blank ultrafiltrates from healthy subjects, diluted 1:1 with MeOH (figure 1). The profiles of the chosen m/z transitions during the course of chromatography were remarkably similar. No drifts or shifts of the selected transition signals were apparent at the retention time of PIs/NNRTIs and I.S. during the chromatography of those blank ultrafiltrate matrices. Co-eluting matrix components may nevertheless reduce or enhance the ion intensity of analytes and affect the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all standard calibrations and quality control samples were prepared by spiking drugs in the same biological matrix (i.e. ultrafiltrate sample diluted 1:1 with MeOH).

Performance of the free drug concentration assays

Precision and accuracy of the LC-MS/MS assay were determined by analysing the QC samples at 2.5, 25 and 200 ng/mL selected to reflect low, medium and high range of the calibration curves. The mean intra-assay precision of the QC samples was 5.0%, 4.1%, 7.9%, 5.1%, 4.1%, 5.4%, 5.2%, 9.8%, 4.8% and 4.2% for APV, ATV, IDV, LPV, NFV, RTV, SQV, TPV, EFV and NVP, respectively. Overall, the mean inter-day precision was good, with average CVs within 4.3-9.3%. The intra-assay deviation (bias) from the nominal concentrations of each analysed antiretroviral drug was between –7.5% and 10.0% and the range of inter-day deviations was always lower than 7.4%. Mean CV% of duplicate determinations in patients (n=296) (reproducibility) were 3%, 10%, 20%, 10%, 12%, 22%, 10%, 16%, and 5%, for APV, ATV, LPV, NFV, RTV, SQV, TPV, EFV, and NVP, respectively.

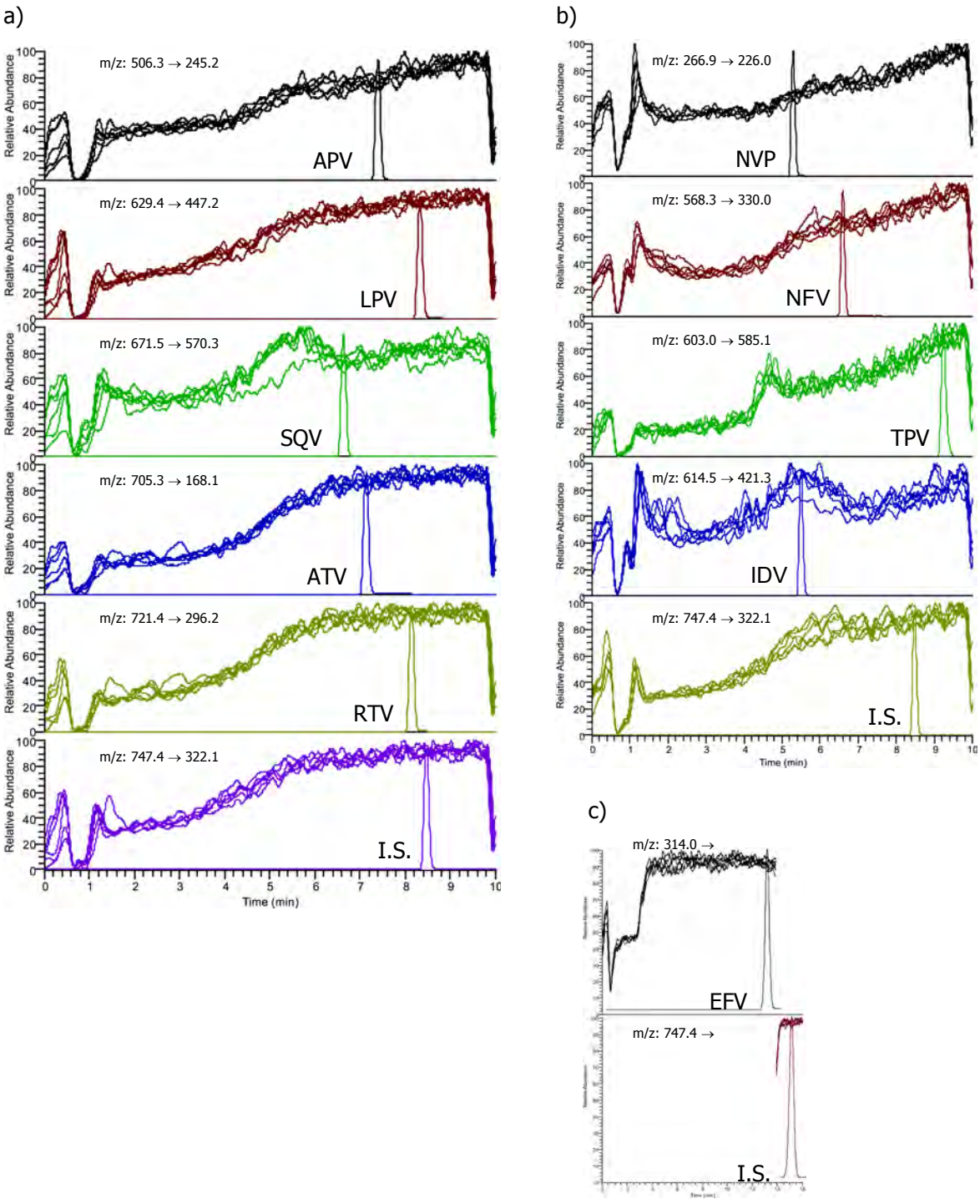

Figure 1: Chromatograms of 6 blank ultrafiltrates with post column infusion of a PIs/NNRTIs solution at 100 ng/mL of each drug and 100 ng/mL of internal standard. Chromatographic profiles for the selected m/z transitions for: a) APV, LPV, SQV, ATV, RTV and I.S.; b) NVP, NFV, TPV, IDV and I.S.; c) EFV and IS.

2.2.4.2. In vitro experiments

Evolution of the ultrafiltrate concentration during ultrafiltration of test samples

Free drug concentrations in the early 0-8 min ultrafiltrate fraction were very low and highly variable (figure 2), with substantially lower free fractions than the later three fractions (8-16, 16-24 and 24-30 minutes) for LPV (mean: 0.89 vs 2.01%, $p<0.05$), NFV (mean: 0.09 vs 0.52%, $p<0.05$), SQV (mean: 0.98 vs 2.73%, $p<0.05$), TPV (mean: 0.014 vs 0.072%, $p<0.05$) and EFV (mean: 0.28 vs 1.17%, $p<0.05$). In the last two fractions, the ultrafiltrate concentrations remained stable, indicating a saturable initial drug adsorption without influence on the accuracy of free drug concentration determination beyond 16 min. The adsorption phenomenon remained modest for IDV (mean: 45.0 vs 52.8%, NS), APV (mean: 18.1 vs 21.3%, NS) and RTV (mean: 6.6 vs 8.8%, NS) and unnoticeable for ATV (mean: 16.3 vs 18.6%, NS) and NVP (mean: 48.2 vs 45.7%, NS). The volume collected in each ultrafiltrate fraction was remarkably similar for all drugs (154 $\pm$ 15 μL (9.8%), 246 $\pm$ 18 μL (7.5%), 302 $\pm$ 19 μL (6.4%) and 337 $\pm$ 19 μL (5.5%), for fraction 0-8 min, 8-16 min, 16-24 min, and 24-30 min, respectively) indicating that the degree of drug protein binding and total and free drug concentrations in the samples had no influence on the ultrafiltrate flux across the membrane.

Comparison of free drug concentrations between standard (0-30 min) and late-only (15-30 min) ultrafiltrate collection

The results presented in table 1 indicate that the 15-30 min fraction collection had higher free drug concentrations for all drugs, except for IDV and NVP. The free fractions in figure 2 are in general agreement with published values, notably those obtained by equilibrium dialysis (1, 17, 32, 34).

Of note, the *in vitro* experiments shown in table 1 and in figure 2 have been performed with *different* batches of blank plasma. The variability in plasma characteristics, notably albumin and AAG levels, explains the difference obtained in the free fractions of the antiretrovirals.

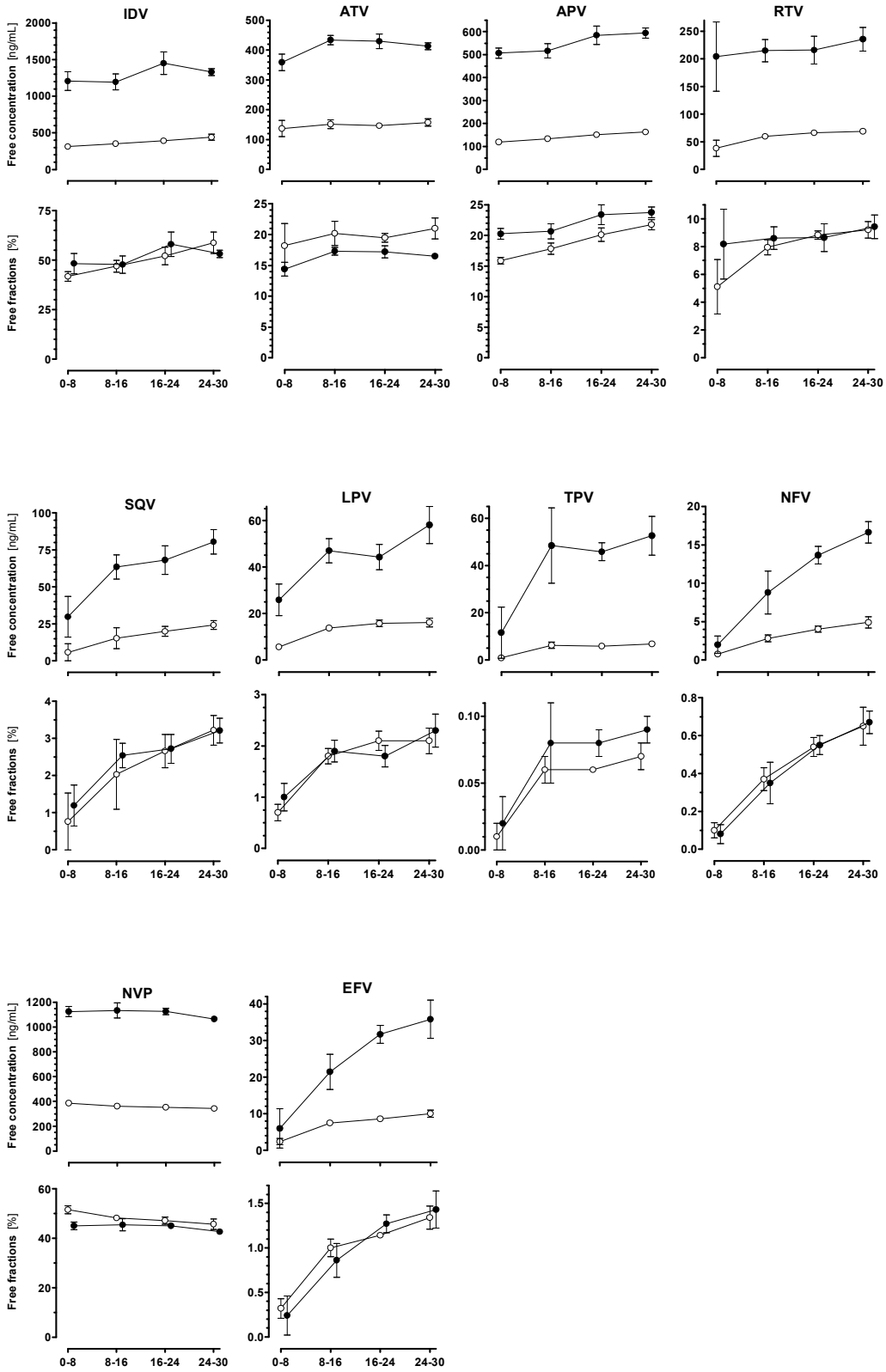

Figure 2: Free concentrations and corresponding free fractions in 0-8 min, 8-16 min, 16-24 min, and 24-30 min fractions.
Open and closed circles correspond to total plasma concentrations of 750 ng/mL and 2500 ng/mL, respectively, for APV, ATV, LPV, IDV, RTV, SQV, EFV, and NVP (10'000 ng/mL and 60'000 ng/mL for TPV).

Table 1: Comparison of free drug concentrations with standard (0-30 min) versus late (15-30 min-only) ultrafiltrate collection

Drug	Total concentration [ng/mL]	Free fraction [%]		Absolute difference	Relative **loss** in free fraction with the 0-30 min method
		0-30 min	15-30 min		
Amprenavir	750	7.3	7.9	0.6	7.5%
	2500	8.3	9.0	0.8	8.4%
Atazanavir	750	14.1	19.6	5.5	28.0%
	2500	13.2	20.1	6.9	34.1%
Indinavir	750	57.4	57.6	0.2	0.4%
	2500	53.0	50.2	-2.8	-5.6%
Lopinavir	750	0.35	0.45	0.10	22.2%
	2500	0.56	0.95	0.39	40.7%
Nelfinavir	750	0.10	0.34	0.24	69.6%
	2500	0.12	0.35	0.23	66.8%
Ritonavir	750	1.6	1.9	0.3	16.4%
	2500	1.8	2.0	0.2	8.4%
Saquinavir	750	1.7	2.9	1.2	41.3%
	2500	1.5	2.7	1.2	43.6%
Tipranavir	10000	0.08	0.09	0.01	13.2%
	60000	0.11	0.13	0.02	15.6%
Efavirenz	750	0.44	1.10	0.66	59.8%
	2500	0.55	1.10	0.55	49.7%
Nevirapine	750	56.0	54.8	-1.2	-2.3%
	2500	60.2	51.6	-8.6	-16.8%

2.2.4.3. Drugs concentrations in patients

Total and free drug concentrations were measured in 151 samples taken at random time after drug intake in 144 patients undergoing routine TDM for APV (n=4), ATV (n=45), EFV (n=51), LPV (n=56), NFV (n=3), NVP (n=20), RTV (n=97), SQV (n=17), TPV (n=3) (296 determinations). The range of plasma total and free concentrations, the mean free fractions with their range and variability (CV%) are shown in table 2.

Table 2: Antiretroviral drugs in blood sample of HIV patients: range of total and free-plasma concentrations; mean free fractions, their range and variability (CV%)

	APV	**ATV**	**EFV**	**LPV**	**NFV**	**NVP**	**RTV**	**SQV**	**TPV**
Total plasma concentration [ng/ml]	303 - 6450	168 - 2940	487 - 9827	677 - 23767	266 - 7266	2306 - 9976	27 - 2251	65 - 4216	11180 - 38735
Free plasma concentration [ng/ml]	11.5 - 324.2	8.1 - 291.7	2.1 - 30.3	4.2 - 209.2	0.3 - 6.6	1015 - 4836	0.1 - 22.8	0.2 - 25.8	0.8 - 32.2
Mean free fraction [%]	4.1	9.6	0.40	0.73	0.09	46.6	0.82	0.76	0.042
Range free fraction (min - max)	3.2 - 5.0	4.8 - 15.0	0.14 - 0.75	0.14 - 1.68	0.09 - 0.10	31.9 - 69.2	0.15 - 1.81	0.22 - 1.57	0.007 - 0.083
Variability free fraction (CV%)	18	25	36	51	5	20	48	59	92
N	4	45	51	56	3	20	97	17	3

Free fractions in patients

Figure 3 shows the free/total plasma concentration ratio (free fraction) plotted against the sampling time after drug intake. No significant time-related trend is observed in the free fraction over the dosing interval (slopes between -0.319 and 0.042 h^{-1}, $r < 0.3$). The small number of measurements available for APV, NFV, and TPV precludes any conclusion.

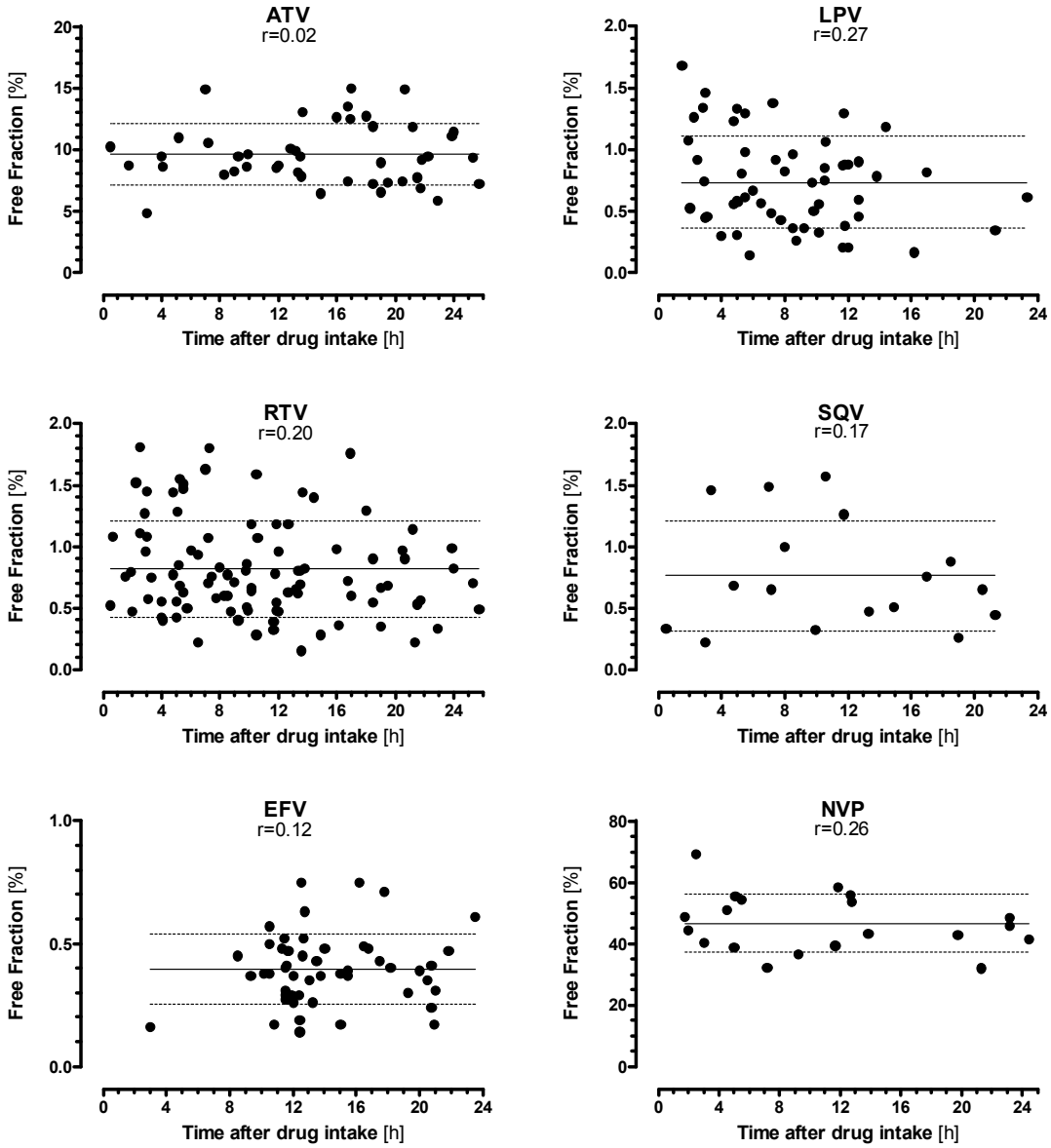

Figure 3: Free fractions over the dosing interval. Full lines and scattered lines represent mean free fraction and 95% confidence intervals, respectively.

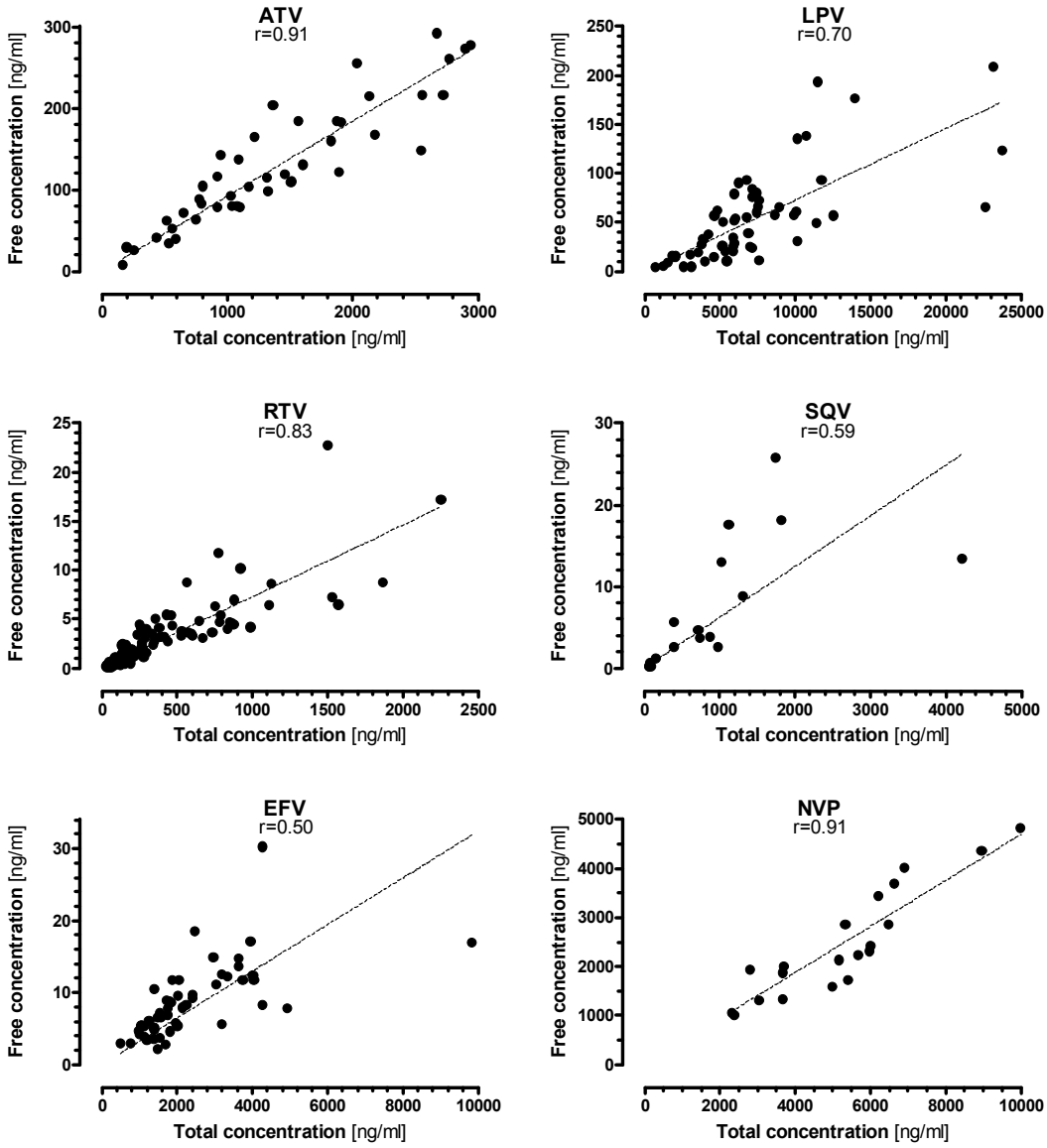

Figure 4: Total versus free drug concentrations

Correlation between free and total antiretroviral drug concentrations

Figure 4 shows the correlation between free and total antiretroviral drug concentrations. The correlation coefficient is 0.91 for ATV and NVP, 0.70 and 0.83 for LPV and RTV, and 0.59 and 0.50 for SQV and EFV, respectively.

Effect of α-1-acid glycoprotein and albumin levels on free fractions

AAG and albumin levels were available for 146 samples, and ranged from 0.21-2.71 g/L and 26.2-60.3 g/L, respectively. The free fractions of antiretrovirals plotted against AAG and albumin levels are shown in figure 5. The influence of AAG and albumin levels on

the relationship between free to total concentrations was also examined for ATV, LPV, RTV, EFV and NVP. Incorporating α-1-acid glycoprotein and albumin plasma levels in the linear regression model resulted in clear improvement of the correlation for LPV (r values: 0.70 $\rightarrow$ 0.92), mostly due to the influence from AAG ($p<0.001$) and to some extent albumin ($p=0.049$). The improvement was moderate for RTV (0.83 $\rightarrow$ 0.89) and ATV (0.91 $\rightarrow$ 0.93), due solely to AAG ($p \leq 0.001$). In contrast, neither albumin nor AAG levels influenced the correlation between free and total concentrations for NVP and EFV.

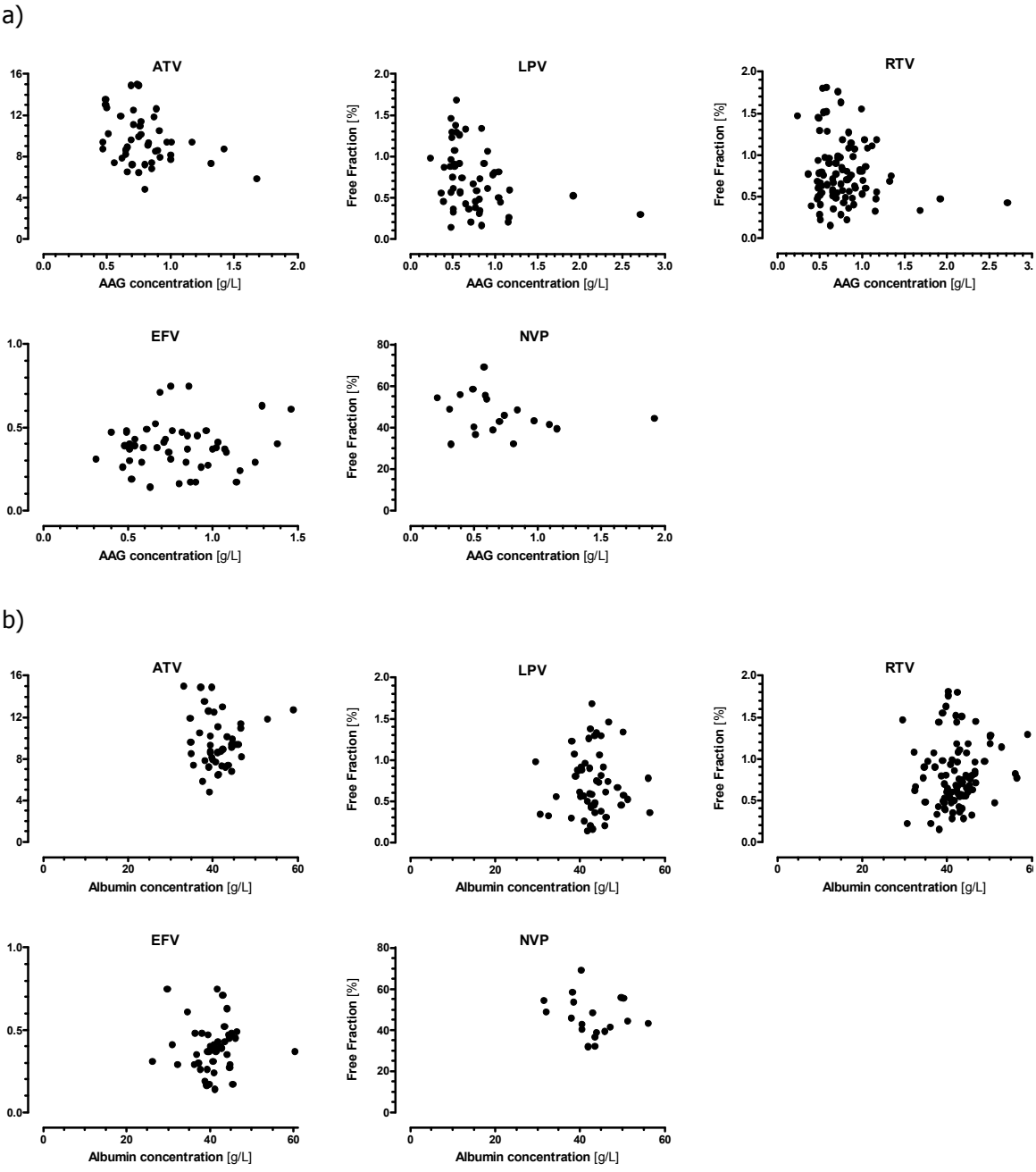

Figure 5: Free fraction plotted against AAG (a) and albumin (b) levels.

2.2.5. Discussion

2.2.5.1. Determination of free drug concentrations by ultrafiltration

The validity of ultrafiltration for the accurate measurement of free drug concentrations has been questioned regularly, since it is not performed under equilibrium conditions and protein concentrates in the retained phase as ultrafiltration proceeds, this effect being even greater near the membrane where plasma proteins accumulate. Following the law of mass action, protein binding of the drug is expected to increase and free drug to decrease progressively during ultrafiltration. This was not observed in our study. Free drug concentrations remained remarkably stable for IDV and NVP – both drugs for which adsorption is not an issue. This confirms earlier observations reported during the determination of phenytoin or valproate free concentrations (52-54). In fact, free protein and protein-bound drug are equally impermeable through the ultrafiltration membrane; they concentrate at the same rate and, therefore, their ratio remains constant. Consequently, the progressive increase in protein concentration does not alter the drug-protein binding equilibrium and the concentration of free drug in the ultrafiltrate, as verified in the rigorous mathematical model proposed by Sophianopoulos *et al* (55).

2.2.5.2. Loss of free drug resulting from adsorption

During early steps of the method development, we observed an important loss of free-antiretroviral drug in the membrane filter and a lack of reproducibility unless the Centrifree® tubes were first washed before plasma ultrafiltration. Therefore, we compared ultrafiltration performance after pre-washing the devices with phosphate buffer, ammonium acetate (5mM, 10mM, 20 mM) and, according to the manufacturer instructions, with purified water and NaOH (0.1N) to reduce interferences between filtered drugs and trace amounts of glycerin in the membrane. The best recovery was obtained, for all drugs, by prewashing the tubes with pure water.

Using test spiked plasma samples with our modified procedure, we noted a marked, albeit saturable adsorption of some ARVs in the first ultrafiltration fraction (0-8 min) and to a lesser extent in the second (8-16 min), without further effect on free drug concentration beyond 16 min of ultrafiltration. This non-specific adsorption phenomenon was especially pronounced for EFV, LPV, NFV, SQV and TPV. Such a mechanism was considered responsible for the underestimation of NFV and LPV free concentrations (17, 22). Loss of EFV during ultrafiltration has also been recognized previously (38). For SQV and TPV, the adsorption issue had never been addressed, but our results suggest that

ultrafiltration may underestimate actual free concentrations. Lower free drug concentrations in the first ultrafiltration fraction have also been reported with the Centrifree® system for other drugs such as phenytoin and valproate (52, 54). Globally however, the importance of early adsorption during the ultrafiltration procedure and its effect on free concentrations has attracted little attention. Adsorption may take place not only onto the ultrafiltration membrane, but also onto the plastic walls of collecting cups. The adsorption on the membrane seems saturable, so can be circumvented by selectively collecting the ultrafiltrate eluted after 15 min (figure 2). The loss of free lipophilic drugs from the ultrafiltrate aqueous environment onto the plastic walls of the cup can be minimized by the rapid addition of MeOH 1:1 w/v to ultrafiltrates, in accordance with previous studies reporting for instance no adsorption of NFV to containers in solutions containing ≥ 35% v/v of acetonitrile or methanol in water (17).

The volume of ultrafiltrate collected in each fraction was similar for all ARVs, regardless of their respective protein binding, and had no influence on free drug determination, in line with the observations of McMillin (53). This indicates that the increase in free drug concentration observed for some drugs during the course of plasma ultrafiltration is not due to a change in membrane efficiency.

Of note, antiretroviral free concentrations determined in our *in vitro* experiments were found to be slightly higher that those in patients, a finding in line with previously reported *in vitro* free fraction values determined in spiked samples (23, 31, 34). Besides intrinsic plasma characteristics, the presence of an organic solvent (MeOH) in the spiked plasma samples may slightly alter the antiretroviral drug-protein binding equilibrium, either by increasing free drug solubility or by modifying plasma proteins conformation. Thus, when *in vitro* ultrafiltration experiments are considered, efforts should be made to limit the percentage of organic solvent added for the preparation of spiked samples as much as possible.

2.2.5.3. Averages values and interindividual variability of free fractions

Our data allowed us to assess the inter-individual variability of free fractions for the major antiretroviral drugs in patients undergoing routine TDM. In this population, free fraction values for ATV, LPV and RTV were higher than those determined in 10 patients included in our previous ATV-LPV/r pharmacokinetic interaction study (25), possibly because loss by adsorption was not fully appreciated at that time. In addition, differences in patient characteristics and concomitant drugs might be involved. The comparison of our free fraction values with available data from the literature indicates

good agreement. For example, the mean LPV free fraction of 0.73 ± 0.37 % found in our 54 patients is similar to the reported value of 0.92 % assessed by Boffito *et al* in 23 patients using the AUC_{free}/AUC_{total} ratio (22). The range of RTV free fraction in our study (0.15 – 1.8 %) also compares well with the range of 0.8-1.6% reported in patients receiving LPV/r (30), but with a higher variability (48%). The range of free fraction values observed in the small number of patients on SQV (0.22-1.57%) is slightly lower than the 0.55-2.7% and 1.1-2.4% reported for SQV, with or without RTV, respectively (28). In the only two available reports on NNRTIs, Almond *et al* (38, 39) have measured median EFV and NVP free fractions (range) of 0.6% (0.4-1.5) and 31.9% (16.2-63.3), respectively, in 10 HIV patients; in our study the corresponding values were 0.40% (0.14-0.75) and 47% (32-69). Our results confirm, on the other hand, the substantial variability in the free fraction previously found for NVP. Finally, the APV free fraction of 4.1% observed in our 4 patients is towards the lower limit of the wide range reported in 10 patients (4.4-20%) (23). To the best of our knowledge, no data have yet been published on the free fraction of TPV, which we found to average 0.042% in 3 patients (range 0.007-0.083).

Despite the large variability observed in the free fractions, both our results and other available data show that free concentrations fluctuate in proportion with total concentrations, and no time-related trend was seen in free fractions over a dosing interval for SQV, IDV and EFV (28, 38), though some small changes in free fraction have been reported for IDV (27) and for LPV (22). Some changes in ATV, LPV and RTV free fractions over a dosing interval were also noted in 10 patients in our previous ATV-LPV/r pharmacokinetic interactions study (25). However, such fluctuations remained quantitatively small. Overall, the protein binding equilibrium can be considered established over a relatively short time, indicating that the dispersion of free fraction values found in the present study do not primary reflect a change over a dosing interval, but mainly the inter-individual variability. For all drugs studied, free to total concentration ratios *versus* time after last dose intake show a homogenous distribution over the entire dosing interval.

2.2.5.4. Correlation between free and total antiretroviral drug concentrations

The correlation can be considered strong for ATV and NVP, moderate for LPV and RTV, and poor for SQV and EFV. For APV, NFV and TPV, free and total plasma concentrations seem well correlated, but the small number of data for these PIs imposes caution in the interpretation of such observations.

The good correlation between free and total drug concentrations for ATV in 45 patients extends and confirms our observation made previously in 10 patients (25). The modest correlation between free and total concentrations for LPV (r=0.70), though confirming our previous findings (25), seems to differs from the only other report published, showing a good correlation between free and total AUCs (r=0.93) (22). Interestingly, the correlation in our study was much improved (r=0.70 $\rightarrow$ r=0.92) by integrating plasma AAG and albumin levels into the model, confirming that these proteins, especially AAG, influence LPV clearance. We also found a poor correlation between free and total concentrations for SQV (r=0.59), in disagreement with a previous report (28). Finally, the correlations between free and total concentrations for NVP and EFV have, to the best of our knowledge, not been reported elsewhere.

2.2.5.5. Effect of α-1-acid glycoprotein and albumin plasma levels on antiretroviral free fraction

Though studies on this issue have been advocated (1, 56) this is the first exploration of the influence of AAG and albumin on free and total drug concentrations in HIV patients. The study of the factors affecting drug-protein binding is important, as protein binding represents a potential variable for the accurate interpretation of TDM data. For example in pregnancy, plasma volume expansion creates a significant degree of dilutional hypoalbuminemia, probably decreasing the binding capacity of plasma albumin, with a consequential increase in drug free fraction. If the increased free fraction leads to a higher free concentration, pregnant women would be expected to have exaggerated drug effects. For most agents (low extraction drugs), changes in protein binding are not likely to alter free concentration profiles and pharmacological effects, as unbound drug determines both the effect and the biotransformation rate and clearance. Clinicians who do not have access to unbound drug measurement may erroneously interpret low total concentrations as indicative of low exposure, and inappropriately increase the doses with a subsequent risk of toxicity (16).

2.2.5.6. Clinical significance of free concentrations of antiretroviral drug

Only if total concentrations correlate well with free concentrations, can monitoring of total plasma concentrations be considered as a valid surrogate for the exposure to pharmacologically active drug. The fact that, for some ARVs, free and total concentrations are only correlated to a limited extent deserves further investigation in terms of clinical consequences. Suboptimal clinical response or unexpected toxicity can

be observed in patients with apparently appropriate total plasma levels: this might be explained in part by free drug concentrations markedly departing from usual values. Facing an unexpected clinical response, knowledge of free/total plasma concentration changes may give useful information. Of note, the Genotypic Inhibitory Quotient (GIQ) or Phenotypic Inhibitory Quotient (PIQ) have been proposed as a monitoring index for PI-based regimens in the guidelines published recently for TDM of antiretroviral drugs (57). In fact, the protein-adjusted IC_{50} proposed at present in PIQ calculations assumes a constant drug-protein binding, without attention to the substantial inter-individual variability in drug-protein binding.

2.2.6. Conclusion

The procedure proposed here for the determination of free drug concentrations of antiretroviral circumvents drug loss due to adsorption of some ARVs onto the membrane and plastic components of the ultrafiltration devices. This method may even be considered for other drugs extensively bound to proteins and adsorbed on ultrafiltration devices.

Applied to samples drawn for routine TDM, this procedure reveals a substantial variability in the free fractions of some ARVs, suggesting free, rather than, total drug concentrations are important, adding an additional level of complexity to the interpretation of total antiretroviral drug concentrations. Since total concentrations may only imperfectly reflect the free, pharmacologically active, concentration, PK/PD relationships may have been obscured by the variability in protein concentration and drug binding. For highly bound drugs (i.e. > 98%, LPV, EFV, SQV), small variations in free drug fraction may have a profound influence on overall exposure to the pharmacologically active drug.

With no controlled studies comparing TDM based on total *versus* free drug measurement, it is premature to recommend performing TDM using free drug concentrations. In special populations or situations, such as pregnancy, altered proteins level, liver insufficiency, or nephrotic syndrome, such an approach may help in clinical decision making.

2.2.7. References

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2.3. Cellular drug concentration

Total plasma concentrations (C_{tot}) are currently measured for Therapeutic Drug Monitoring (TDM). However, except entry inhibitors (maraviroc and enfuvirtide), which act on receptors located on the cell surface, the site of antiretroviral activity of ARVs is inside the cells. Thus, the measurement of C_{tot} may not perfectly reflect the intracellular drugs levels where ARVs are expected to exert their pharmacological activities. In previous *in vitro* studies (1,2), the antiviral activity of PIs was shown to be highly correlated with the intracellular drug concentration in cell lines, which has formed the rationale for the development of *in vivo* studies. Therefore, the study of intracellular PK may provide information regarding drugs in a compartment where HIV replication occurs and, combined with plasma data, may help to understand some instances of therapeutic failure and cellular resistance (3).

The available evidence shows that the homeostatic control of intracellular concentrations of ARVs is complex and depends on many factors, such as drug's physicochemical properties (lipophilicity, degree of ionisation), plasma and cellular protein binding, or activity of influx and/or efflux transporters whose expression and activities may be influenced by genetic polymorphism, or by coadministration of drugs with inhibiting or inducing properties (3,4). The ultimate disposition of ARVs within cells remains unknown, but a dynamic equilibrium may exist between unbound and protein-bound fractions, influenced by the affinity of the drug for each protein (4).

Isolation of peripheral blood mononuclear cells (PBMCs) is the first step in analysis of intracellular concentrations of ARVs. PBMCs can be isolated using either conventional Ficoll® gradient centrifugation or Vacutainer® cell preparation tubes (CPT). These two procedures have been previously compared and have shown to give identical results (5). In our case, the CPT method was retained, because easier to use and less time consuming (see Appendix 6.2).

The total amount of antiretroviral drugs contained in cells is a function of the number of cells collected from patients at each blood sampling. A necessary step implies therefore the determination with a Coulter instrument of the number of cells contained in each patient's PBMCs pellet into which drug are quantified. The concentration, expressed as the amount of drug per 10^6 cells, can be converted into the amount per volume, assuming a PBMC volume of 0.4 pL. However, this approximation may not reflect the

volume of intracellular liquid medium for which the free drug species is available, as cell volume may vary according to the state and the nature of the cells. But to date, the volume of a single cell remains the best surrogate marker to calculate intracellular concentrations and is most commonly used (3,4). It must be acknowledged that in all current studies on intracellular disposition to date, the so-called intracellular drug concentrations should be considered as cell-associated drug amount. The currently published intracellular assays do not differentiate between free drug truly soluble in cell cytoplasm, and bound to cell components (membranes, cell proteins).

Most reported data on the measurement of intracellular concentrations of PIs and NNRTIs were obtained using the tandem mass spectrometry technology (6-13). In the present work, we have adapted our LC-MS/MS methodology developed for plasma assay of the recent ARVs (14) to reach the high sensitivity required for measuring the intracellular concentrations of raltegravir, darunavir, etravirine and maraviroc in HIV-infected patients (see Chapter 6.1. and Appendix 6.3).

At present, intracellular accumulation data are usually quantified and expressed as a ratio of the intracellular area under the curve (AUC) over the total plasma AUC throughout the dosage interval (3) as an index of exposure in the respective compartment. Indeed, plasma and intracellular half-lives may be different for some drugs, that may possibly influence the results when studying relationships between plasma and cell concentrations measured at single time-points (4). Another approach would be the use of population pharmacokinetic analysis of intracellular concentrations, but would necessitate a large number of data, which may not be always available given the logistical and technological difficulties to perform cellular pharmacokinetics in vivo. (see Chapter 2.5).

As intracellular concentrations of current PIs and NNRTIs have been extensively studied, we decided to focus on recent antiretroviral drugs: raltegravir, maraviroc, darunavir and etravirine, for which cellular PK remains a largely unexplored field of investigation that may potentially be of clinical relevance in the context of viral resistance and treatment failure in the few patients who do not respond well to these new agents.

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2.4. Metabolite profiling

Up to now, most investigations on the clinical pharmacology of antiretroviral drugs have focused almost exclusively on the TDM of parent drugs in plasma, and their potential PK/PD relationships, considering the parent drug as the best pharmacokinetic marker of antiretroviral drug exposure, and, in case of high levels, of toxicity. However, drug metabolites resulting from complex mutual genetic and environmental influences could play an important role in the toxicity, tolerability and treatment outcome of ART.

The *metabolite profile* can be considered as a snapshot on the phenotypic pattern of the metabolising activity in a patient at a given time. The most prominent example of the interest to focus on metabolite profiling comes from the field of oncology, with the tamoxifen, a hormone therapy used for prevention of breast cancer relapse. After nearly 30 years of a wide use of tamoxifen, it was only recently recognised that most of its antioestrogenic activity is due to minor metabolites (4-OH- and desmethyl-4-OH-tamoxifen) that have 30-100 times more affinity for the estrogen receptor than tamoxifen itself (1). As tamoxifen is converted into active metabolites by CYP2D6 and CYP3A4/5, genetic background and environmental influence after the active metabolites blood levels, and thus clinical outcomes (2,3). Therefore, patient genotyping, and possibly, tamoxifen metabolites monitoring, could significantly improve clinical efficacy.
Whereas labelling information generally reports for most antiretroviral drugs the presence of several metabolites in humans, there are very few published articles describing in some detail the metabolites profiling of first generation and more recent antiretroviral drugs. Most studies were restricted to the influence of CYP2C19 polymorphism on nelfinavir to its active metabolite M8 biotransformation (4,5), and *in vitro* (6) or *in vivo* (7) studies on the influence of CYP2B6 polymorphism on the rate of oxidative metabolism for efavirenz.

Nevertheless, with access to the current LC tandem MS technology, the simultaneous measurement of the parent drug and its metabolites can be performed in individual's plasma samples. Patients' metabolites profiling constitutes useful additional information for a comprehensive analysis of pharmacokinetic-pharmacogenetic relationships.

As an example, during this thesis we have developed a new LC-MS/MS method applied for the efavirenz metabolites profiling of its main accessory metabolic pathways (see §.5.3.), thus allowing the simultaneous detection of efavirenz (EFV), 7-OH-efavirenz (7-

OH-EFV), 8-OH-efavirenz (8-OH-EFV) and efavirenz-N-glucuronide (EFV-N-Gln) in small volumes of plasma from patients (Figure 1).

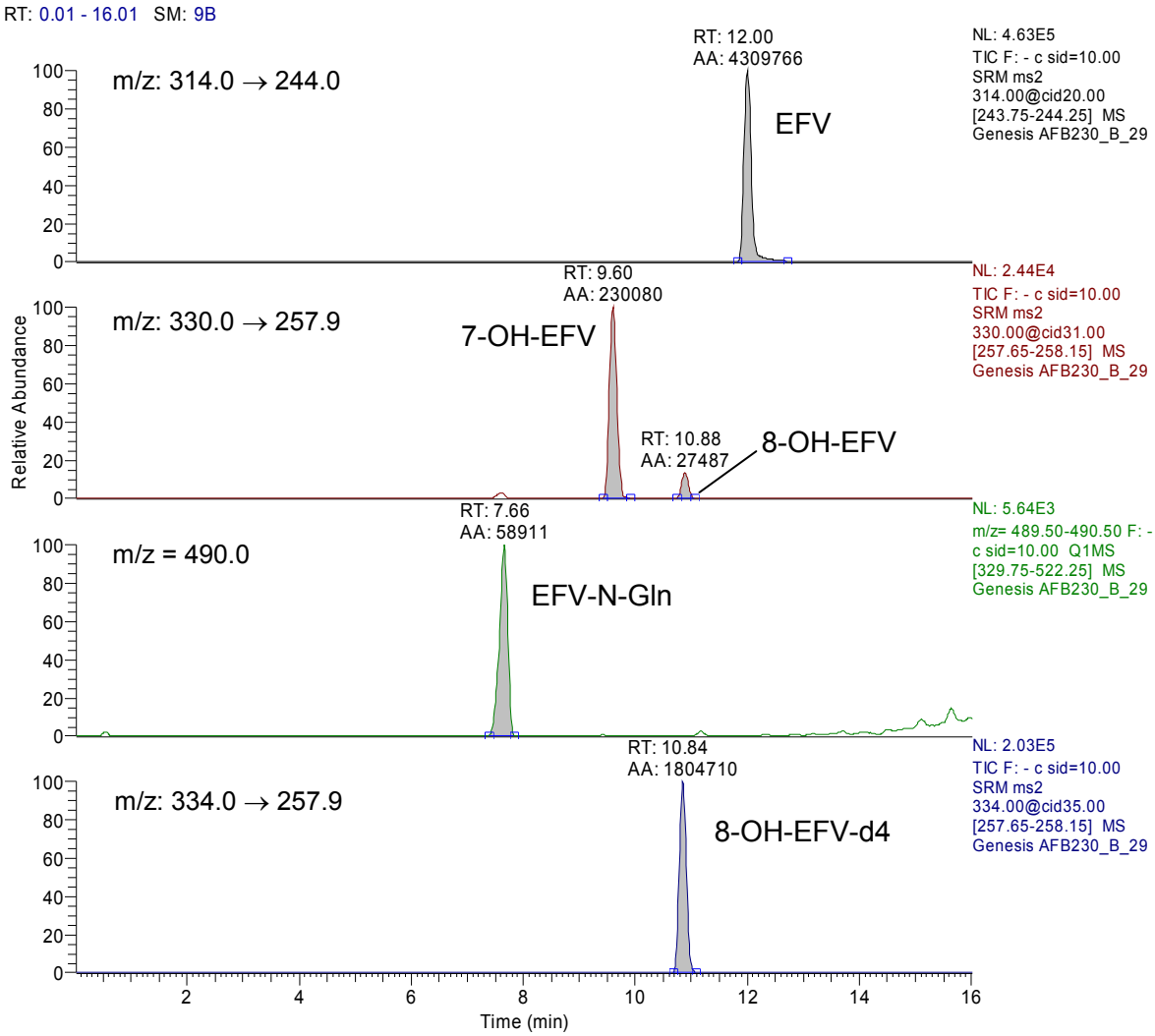

Figure 1: Typical efavirenz metabolites profiles analysis in a selected patient on efavirenz-based regimen.

The *selected reaction monitoring* (SRM) mode enables to detect with high specificity a distinct molecule for each transition requested, as for EFV (parent mass 314.0 → product mass 244.0), whereas the *full scan Q1 MS* mode allows the identification of all molecules present in sample that have a parent mass of 490.0 amu for example, corresponding to the addition of one glucuronide at N-position.

Similar metabolite profiling approach has been used to identify in patients the raltegravir glucuronide reported to be the main raltegravir metabolite in humans (see §.2.1.)

2.4.1. References

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2.5. Population pharmacokinetics and NONMEM®

2.5.1. Population pharmacokinetics

Population pharmacokinetics (PopPK) is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant dose of a drug of interest (1). PopPK seeks to identify the measurable pathophysiological factors that cause changes in the dose-concentration relationship and the extent of these changes. If such changes are associated with clinically significant shifts in the therapeutic index, dosage can be thus appropriately modified. Its advantages over more traditional PK characterisation include the actual study of the population of interest, with the possibility of sparse random time sample collection, better estimates of population means and variances, opportunities to assess multiple factors that may influence drug disposition and at least a theoretical cost-benefit advantage (2).

This approach will ensure an appropriate dosage adjustment to accommodate clinically significant differences in pharmacokinetics due to genetic, environmental, physiological or pathological factors. Therefore, PopPK models generally using Bayesian approaches for estimating individual pharmacokinetic parameters play a pivotal role for patient care both during early drug's clinical development, as well as in post-marketing studies, as part of TDM, by optimising patient dosing strategies (see § 2.6).
There is currently an increasing interest for the implementation of PopPK approach that is now an integrated part of drug development (3). In that context, FDA has published guidance for industry on the use of PopPK to help identify differences in drug safety and efficacy among population subgroups (4).

While structural PK/PD models have been built and used to generate individual patient PK/PD parameters, population behaviour has often been estimated *via* simple descriptive statistics or ANOVA (analysis of variance) with linear approximation employed. A population-based approach reliant on nonlinear mixed-effect modelling is clearly superior, especially when data are sparse, unbalanced and fragmentary. Moreover, it has the potential to reduce the frequency of blood sampling required for estimating PK parameters (2,4).

2.5.2. NONMEM®

A specialised software, called NONMEM® (for nonlinear mixed effects model), was developed by the NONMEM Project Group at the University of California at San Francisco (5,6). The NONMEM® program aims at providing methodological guidelines and computer tools for the analysis of data that may be described by regression-type models, and was developed for analysing population pharmacokinetics in particular. Proper modelling of these data involves accounting for both explicit factors (fixed effects) and unexplainable inter- and intra-subject effects (random effects). It is a general program, especially useful when few PK measurements are available from each individual, or when the data collection design varies considerably between individuals. However, it can be applied to fit models to a wide variety of data.

The general expression of a model is:

$$y_{ij} = f(x_{ij}, \phi_j) + \varepsilon_{ij} \qquad \text{with} \quad \phi_j = g(z_i, \theta) + \eta_j$$

where y_{ij} is the i^{th} observation from the j^{th} individual, f is a specified pharmacokinetic (or even pharmacodynamic) model, which is function of known quantities x_{ij} (e.g. dose, time) and parameters ϕ_j (e.g. clearance, distribution volume). The parameters ϕ_j are function of fixed effects z_i (e.g. age, height, genotype, co-medication) and fixed effects parameters θ. The random effect in the residual errors is denoted by ε and the inter-individual error by η_j.
This general model has thus (i) a structural part $y_{ij} = f(x_{ij}, \phi_j)$ and $\phi_j = g(z_i, \theta)$, function of a number of constants (fixed effects parameters and covariates), and (ii) a probabilistic part $\hat{y}_{ij} = y_{ij} + \varepsilon_{ij}$ and $\hat{\phi}_j = \phi_j + \eta_j$, made of two random effect parameters (inter-subject and residual variabilities) (7,8).

The pharmacokinetic model is to be selected among classical PK models such as one or two compartments, linear or nonlinear kinetics for PK variables (e.g. drug concentration). By a maximum likelihood approach, NONMEM® program aims to determine the best model to fit the analysed data estimating the average values of θ, ω^2 and σ^2 which give the lowest value of an objective function (OF). The value of the OF is used to compare two models applying the likelihood ratio test. The statistical theory indicates that a decrease of 3.8 points in the minimum value of OF is significant at $p<0.05$ for one additional parameter (9).

To date, several PopPK models have been published for most PIs and NNRTIs (10-16), but it remains largely unexplored for more recent antiretroviral drugs (17).

More generally, the clinical usefulness of the PopPK approach has been acknowledged in medicine and in clinical pharmacology, allowing the possibility for Bayesian feed back adjustment of dosage regimens, which represents the best currently available therapeutic drug monitoring strategy.

2.5.3. References

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2.6. The Bayesian approach

An accessible approach to interpret random plasma drug levels from patients is based on a Bayesian population pharmacokinetic model (commonly called "*poor man's population pharmacokinetics*"). This latter predicts individual PK parameters by applying a previously established mathematical model on a single pharmacokinetic patient's information and balancing the result against the predicted population values.
The Bateman equation is used to calculate predicted concentration for each individual PK parameter, based on population PK parameters:

$$C_{pred} = \frac{F \times D \times k_a}{V \times (k_a - \lambda)} \times \left(\frac{1}{1 - e^{-\lambda\tau}} \times e^{-\lambda t} - \frac{1}{1 - e^{-k_a\tau}} \times e^{-k_a t} \right)$$

where C_{pred} = predicted concentration for the individual [ng/ml]; F = biodisposition of the drug; D = dose of the drug administered [mg] ; V = population distribution volume of the drug [L]; k_a = population absorption constant [h^{-1}]; λ = time constant (= CL/V, clearance over distribution volume) [h^{-1}]; τ = time interval between 2 doses [h] ; t = time interval between last drug intake and blood sampling [h].

An objective function (Φ) is then set out, allowing the research of the most likely PK parameters (CL, V, k_a) for the individual by minimisation of this function:

$$\phi = \sum \frac{\left[\log(\theta_{ind}) - \log(\theta_{pop})\right]^2}{\omega^2} + \frac{\left[\log(C_{obs}) - \log(C_{pred})\right]^2}{\sigma_{prop}^2} + \frac{\left(C_{obs} - C_{pred}\right)^2}{\sigma_{add}^2}$$

where θ_{ind} = individual PK parameters (CL, V, k_a); θ_{pop} = population PK parameters (CL, V, k_a); ω = variability on PK parameters (CL, V, k_a); C_{obs} = random observed concentration [ng/ml]; C_{pred} = individual predicted concentration [ng/ml]; σ_{prop} = residual proportional variability in the plasma concentrations [CV %]; σ_{add} = residual additive variability in the plasma concentrations [SD ng/ml].

This process essentially shows how different from the population a given individual is, and allows the determination of drug exposure (e.g. AUC, area under the curve) from a random observed drug plasma levels (C_{obs}), by also including all covariables from the

individual that have been shown to influence the model. It is therefore possible to extrapolate from sparse plasma samples collected at unselected (random) time various important pharmacokinetic parameters, such as overall exposure to the drug (AUC) and minimum concentration at the end of dosing interval (C_{min}), for example. This PopPK approach is in fact required for performing a valid comparison of extrapolated AUC and C_{min}.

This Bayesian approach will be used in Chapter 4.1 for the pharmacokinetic analyses of a clinical study in which a limited number of blood samples were taken in HIV-positive pregnant patients at random times over the entire course of pregnancy.

2.7. Pharmacogenetics

The terminology pharmacogenetics was coined by Vogel in 1959 (1), to mean "knowledge of inherited variants with regard to the reaction to pharmaceuticals and other administered compounds". Pharmacogenetics focuses on the effect of a single gene on drug response, while pharmacogenomics deals with the effects of multiple genes on drug response. However, both terms are currently used interchangeably.

Genetic variability in drug response occurs as a result of molecular alterations, such as gene deletion, single nucleotide polymorphism (SNP) or gene duplication, at the level of drug-metabolising enzymes, drug targets/receptors and drug-transport proteins (2). Thus, all aspect of drug disposition (absorption, distribution, metabolism and elimination), as well as drug targets themselves, constitute the domain of the pharmacogenetic approach. Depending on the nature of the genetic variation and its location within a given gene, the phenotypic consequences may be barely noticeable, or may alter the function or expression of the encoded protein. Because these variations can generate changes in the function of proteins that interact with a drug, the response to a drug may differ among individuals. Understanding how these variations influence drug response may certainly have implications that would help in the monitoring of ART (3,4).

Despite 50 years of history, most of the progress in pharmacogenetics has been made in recent years, thanks to the driving force of the Human Genome Project and the International HapMap project, along with the rapid development of advanced genetic technologies (3).

At present, two most common strategies are used for gene-disease and gene-drug response association studies: the candidate-gene approach and the genome-wide association study (GWAS). The former is a useful tool to tests whether a particular allele or a set of alleles is more frequent in patients who have a better – or worse – drug response. In this case, the knowledge about the function of a gene is essential for selecting a gene to study. The GWAS examines the common genetic variations for a role in disease or drug response by genotyping large sets of SNPs across the genome. This is a great tool to discover new functions of a gene or to identify a new genetic biomarker that may be used as a surrogate for drug response. Unlike the candidate-gene approach, GWAS is more expensive and requires a large clinical sample size, factors that limit its use (3).

A number of associations have been reported between host genetic polymorphisms and response to ARV drugs (5-7). The best example of casual genotype-phenotype correlation is the association between human leukocyte antigen (HLA) type HLA-B*5701 and abacavir (ABC)-associated hypersensibility reaction syndrome (8). HLA-B*5701 testing has already entered the routine clinical practice as the standard of care before ABC prescription. Other well-established associations are the role of CYP2B6 alleles in EFV pharmacokinetics and toxicity (9), or the association of UGT1A1 polymorphism in atazanavir (ATV)-associated hyperbilirubinemia. In these two cases, genotyping for *CYP2B6* and *UGT1A1* have been proposed for the treatment individualisation of EFV and ATV, respectively.

In fact, HIV infection and treatment represents an important field for application and validation of pharmacogenetic knowledge. In Switzerland, this field has excellent patient cohorts and well-developed structures for clinical trials that could allow pharmacogenetic investigations. However, numerous barriers exist to the direct translation of this knowledge toward therapy individualisation: risk of false discoveries caused by multiple testing, small study size, lack of adequate statistical power, presence of selection bias. Thus, several preconditions exist for successful introduction of a pharmacogenetic test into routine clinical practice. The test should be rapid and simple to interpret, with high sensitivity and specificity. Moreover, randomised clinical trials are needed to evaluate the clinical utility of a pharmacogenetic test. Although pharmacogenetics tests may help inform clinical decisions involving drug selection or dosing, it has not been shown whether these tests improve clinical outcomes. Finally, robust cost-effectiveness data should be provided to support its reimbursement by insurances and sustainability by healthcare systems (3,5).

Definitely, pharmacogenetics of ARV drugs could help to find the best tolerated combination of drugs, and predict a treatment regimen that will reach "ideal" concentrations in order to prevent the emergence of resistance and avoid toxicity, toward the ultimate goal of personalised ARV therapy. But it will be more challenging than anticipated originally, particularly with regard to the clinical validation of genetic findings and their translation into medical tools.

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PART III

CLINICAL APPLICATIONS

Chapter 3:
Therapeutic Drug Monitoring

The treatment of HIV-1 infection requires the availability of a number of diagnostic assays helping the clinicians to optimise an individualised treatment for their patients. While determination of the optimal dosage for a given drug is generally based on dose-clinical response relationships (e.g intensity of viral drop, CD4 increase, or tolerability and convenience), evidence of the link between ARV drug exposure and antiviral efficacy or toxicity has begun to focus attention on the role of monitoring plasma drug levels in patients receiving HAART.

Therapeutic Drug Monitoring (TDM) involves the determination of total plasma drug concentrations and an expert pharmacokinetic interpretation of the result to detect unsatisfactory drug levels and to adequately correct for drug dosage, taking into account viral load, immunology, resistance tests, data on adherence, comedications, occurrence of adverse effects, and with the ultimate goal of an individualisation of patient antiretroviral regimen.

Among antiretroviral classes used clinically, a TDM of PIs and NNRTIs appears to present some clinical benefit for the management of antiretroviral treatment. Indeed, numerous studies showed association between drug exposure and virological outcomes (1-5), and conversely, on short or long-term toxicities (1,2,6,7) or virological failures (5). More generally, general correlation between ARVs plasma levels and virological efficacy is certainly true in the large sense as short-term compliance and true pharmacokinetics problems (eg, poor plasma levels at the end of the dosing interval) can certainly be identified.

Apart from these concentration-response relationships, PIs and NNRTIs present a large inter-individual variability generally coupled with a low intra-individual variability, characteristics that are necessary to be considered an appropriate candidate for drug monitoring. For clinicians, maintaining individual patients ARV plasma concentrations within a therapeutic range by drug monitoring is an important addition to CD4 cell count and viral load for improving HIV-1 management.

However, despite its intuitive potential utility, the role of TDM remains to be clearly established in the setting of HIV-1 management. Carefully conducted randomised controlled trials evaluating the benefit of TDM for ARVs are lacking. In fact, a recent Cochrane review (8) concluded, after a systematic review and meta-analysis of 8

available randomised studies (9-16), that there is a lack of support for routine use of TDM of ARVs.

The debate regarding the clinical utility of ARV TDM is also reflected by the conflicting recommendations in international HIV management guidelines. Thus, the World Health Organisation guidelines for adults and adolescents of resource-limited settings do not recommend ARV TDM for routine use in the management in HIV-infected adults (17). Guidelines from the U.S. Department of Health and Human Services (DHHS) are supportive of ARV TDM without providing specific recommendations (18). The British HIV Association supports the use of TDM in specific clinical situations including pregnancy, paediatrics, management of drug interactions, hepatic or renal dysfunction, or in cases with toxicity or new regimens whose efficacy and safety are not yet well defined (19). Besides this, none of the guidelines specify recommendations for the implementation of TDM (8), and a number of issues have to be addressed: what are the appropriate time points or interval for testing; what are the appropriate therapeutic ranges or target concentrations, what is the best time for blood sampling; or what tools and covariables are to be used for interpretation and for the formal drug dosage adjustment in patients.

In the meantime, unofficial guidelines for ARV TDM from panel of researchers and opinions leaders in the field become increasingly frequent, like the *Updated guideline to perform TDM of ARV agents* recently published in 2006 after the 7th International Workshop on Clinical Pharmacology of HIV Therapy (20).

In fact, a large-scale, prospective, randomised and sufficiency-powered trial still remains a need to evaluate the clinical usefulness of ARV TDM, but seems to be difficult to implement in countries already incorporating TDM into their routine HIV management, as in Switzerland, France, the United Kingdom or the Netherlands for example (8).

Meanwhile, TDM of ARVs is already largely used and appreciated by clinicians throughout Switzerland. In this field, the Division of Clinical Pharmacology at CHUV is progressively being recognized as the reference centre in Switzerland, notably because of our growing expertise in pharmacokinetics expert interpretations and mass-spectrometry analysis. Of note, the pharmacokinetic data generated by our laboratory are prospectively integrated into the Pharmacology database of the Swiss HIV Cohort Study, for subsequent Swiss-wide population pharmacokinetic analysis. Importantly, laboratories performing TDM should participate to external quality control programs to ensure the validity of the results. Our laboratory participates actively to the European program for TDM in HIV Infection provided by the Netherlands KKGT Association (21).

Finally, whether TDM would be also beneficial for new classes of antiretroviral drugs, such as the new integrase inhibitor raltegravir and the CCR5-coreceptor antagonist maraviroc, as well as for the next-generation PI darunavir and NNRTI etravirine, remains to be established. In fact, pharmacokinetics for these recent antiretroviral drugs is a largely unexplored field of investigation. All these new agents represent significant therapeutic advances, as they have new mechanisms of action or exhibit excellent activity against viruses resistant to other agents of the same class. However, many of them appear to display important pharmacokinetic complexities in terms of drug-drug and drug-food interaction and very large interpatient variabilities that can complicate management of treatment (22-24). Therefore, these new agents can be predicted to also represent valid candidates for rational therapeutic individualisation strategies.

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Chapter 4:
Optimising ART in specific conditions

A defining feature of the pandemic in the current decade is the increasing number of HIV-1 infections in women, which has additional implications for mother-to-child-transmission (MTCT). By the end of 2008, approximately 15.7 million women were living with HIV/AIDS worldwide. An estimated 430'000 new HIV infections occurred among children under 15 years in 2008. Most of these new infections occurred during the course of pregnancy (*antepartum*), around the time of labour and delivery (*intrapartum*), or *postpartum* through breastfeeding (1).
In low resource settings, where access to antiretroviral therapy is limited and breastfeeding prolonged, the MTCT rate is 30-35%, HIV remaining a major cause of mortality and morbidity (1,2). By contrast, in the developed world, the use of combination ART, elective caesarean section and exclusive formula feeding, has become the standard of care for prevention of mother-to-child transmission (PMTCT), and resulted in transmission rate of less than 2%, almost eliminating vertical transmission of HIV (2). Indeed, ARVs have been used routinely to reduce the risk of MTCT since 1994, following the positive results of the first trial evaluating the use of zidovudine monotherapy as an intervention for the PMTCT (3).

Beside treatment of the mother's underlying HIV disease, HAART is now the standard of care to prevent mother-to-child HIV transmission, its benefit in pregnancy clearly outweighing the potential risk to the foetus. Moreover, since a low HIV viral load in pregnant women is a powerful predictive factor of MTCT, the main goal of HAART during pregnancy is to maintain undetectable levels of HIV RNA in order to prevent MTCT (4,5).

However, there have been several reports of reduced ARVs exposure during pregnancy that have prompted some, but not all, groups to increase ARVs dosage in pregnant women. In fact, as HIV-infected pregnant women are generally excluded from clinical trials the impact of pregnancy on ARVs disposition is imperfectly known but this is an issue that definitely deserves further evaluation. Within the frame or our work on antiretroviral therapy optimisation, we have therefore initiated a prospective observational study on the pharmacokinetics of antiretroviral drugs in pregnant HIV positive patients followed over the entire course of pregnancy.

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4.1. Free and total plasma levels of lopinavir, atazanavir and nevirapine during pregnancy, at delivery and in postpartum: dosage implication for pregnancy

Therapeutic Drug Monitoring (TDM) of NNRTIs and PIs is probably justified during pregnancy to ensure that plasma concentrations of ARV are sufficient during the whole course of pregnancy, as ARVs exposure are obviously dependant of strict compliance to treatment, that may not be perfect during pregnancy, or by true pharmacokinetics problems possibly modified by specific physiological changes in maternal pharmacokinetics. Indeed, pregnancy may affect the absorption, distribution, metabolism and elimination of ARV drugs, and consequently may influence the potential efficacy and/or safety of the ARV treatment for the mother and her child.

At the moment, it is still unclear whether these physiological changes need to be taken into consideration for antiretroviral dosage individualisation in HIV-positive pregnant women. Moreover, studies on TDM during pregnancy have at present only focused on *total* plasma concentration, whereas antiviral activity is expected to depend on *free* concentrations. However, pregnancy is accompanied with changes in plasma proteins composition, likely to alter drugs protein binding. TDM of free – unbound – concentrations may be therefore more relevant that TDM based on total drug concentrations.

To answer this clinically important issue, we have initiated a prospective observational study that aims at measuring simultaneously free and total drug concentrations of protease inhibitors and nevirapine in HIV-positive pregnant women during the whole course of pregnancy and at postpartum, in a prospective Swiss-wide survey (SHCS #469). Free and total placental transfers of these drugs have been also examined.

Related Appendices: 4.1, 4.2, 4.3

FREE AND TOTAL PLASMA LEVELS OF LOPINAVIR, ATAZANAVIR AND NEVIRAPINE DURING PREGNANCY, AT DELIVERY AND IN POSTPARTUM: DOSAGE IMPLICATION FOR PREGNANCY

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(submitted)

4.1.1. Abstract

Whether physiological changes associated with pregnancy influence antiretrovirals exposure is still controversial. We described free and total pharmacokinetics of lopinavir, atazanavir and nevirapine in HIV-infected women during pregnancy, at delivery, in umbilical cord and in postpartum, and evaluated whether significant alterations in their disposition and protein binding warrant systematic dosage adjustment. Free and total concentrations were measured by LC-MS/MS in plasma from 61 pregnant and 68 non-pregnant women. Total lopinavir exposure was minimally (-11%) decreased, whereas free lopinavir exposure was not influenced by pregnancy. For atazanavir, a slight not significant decrease of total and free exposure (-27%) was observed compared to postpartum. For nevirapine, neither total nor free exposure was significantly modified by pregnancy. Free cord-to-mother ratios were significantly higher than total cord-to-mother ratios for lopinavir and atazanavir, suggesting higher fetal exposure. Thus, during pregnancy, exposure to free lopinavir, atazanavir and nevirapine remains unaltered. No dosage adjustment is therefore needed.

4.1.2. Introduction

Mother-to-child transmission (MTCT) of Human Immunodeficiency Virus (HIV) is the primary cause of paediatric HIV infections in the world, contributing to infant and child mortality and highlighting the importance of prevention. Available data suggest that *in utero*, HIV transmission is rare, while up to 75% of MTCT occurs during or after delivery. Advances in antiretroviral treatment and specific obstetrical procedures have markedly reduced the risk of virus transmission through child-bearing (1).

Compared to vaginal delivery, elective Caesarean section performed prior to labor and with intact membranes decreases MTCT by 50%. It has been recently suggested, however, that in women successfully treated with highly active antiretroviral therapy (HAART) having low (< 1000 copies/ml) or undetectable viral load, the potential side effects of Caesarean section might outweigh its benefits regarding MTCT (2). Indeed, maternal plasma viral load is the strongest independent predictor of MTCT (2), and HAART can effectively control viral replication and reduce the risk of transmission down to 1 to 2% (3).

The benefit of treatment must also be balanced against potential adverse effects on pregnancy, the embryo and the foetus (2), such as anaemia, low birth weight, prematurity (4), spontaneous abortion, stillbirth or induced abortion (5). Efavirenz is teratogenic in animals (6). Based on current evidence, the benefit of the other antiretroviral agents (ARVs) in pregnancy is considered to outweigh their potential risks to the foetus (7-8).

Physiological changes occurring in the maternal-placental-foetal unit during pregnancy may alter the processes of drug absorption, distribution and elimination of ARVs (9,10) and influence their efficacy and safety. Pregnancy affects variably the presystemic and liver metabolism of drugs, and increases their renal excretion, thus justifying recommendations to monitor plasma concentrations whenever possible (11). Drugs are circulating in two forms in plasma: a fraction bound to plasma proteins (mostly human serum albumin (HSA) and α-1-acid glycoprotein (AAG)) and the remainder unbound (free). Unbound molecules diffuse into tissues and penetrate into cells to exert their activity, while the total (free + bound) concentration is usually determined for Therapeutic Drug Monitoring (TDM) (12,13). Wide differences exist in the binding of ARVs. Protease inhibitors (PIs) are mostly lipophilic weak basic molecules highly bound to plasma proteins (> 85%, except indinavir 60%), mainly to AAG. The weakly acidic

non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine bind predominantly to HSA (> 99% and 60% respectively) (14).

A small change in the extent of protein binding of highly bound drugs may result in a significant effect on the free fraction (free/total concentrations ratio) (15). Besides diseases or infections, pregnancy also affects drug protein binding (15-19). This may confuse the interpretation of TDM results based on total concentration measurement. For drugs of *low* hepatic extraction, a change in proteins concentration or in binding affinity to HSA or AAG alters *total* plasma concentrations, while *free* drug levels remain mostly unchanged (20). Conversely, changes in protein binding for drugs of *high* hepatic extraction are not expected to reflect on *total* drug concentrations, while *free* drug levels are actually affected. In both situations, an altered free fraction impacts on apparent total concentration-effect relationships and may compromise the interpretation of TDM results. Unbound drug concentration determination might therefore provide a more accurate indicator of "effective" drug exposure and contribute to more appropriate dosage recommendations and individualization in pregnant patients (14,15,21).
In this study, we determined the total and free plasma concentrations of common ARVs during pregnancy, at delivery and in postpartum, to evaluate whether significant alterations in their disposition and protein binding warrant systematic adjustment of dosage or specific monitoring procedures.

4.1.3. Results

4.1.3.1. Patients

One hundred and twenty-nine HIV-infected women were enrolled in the study (Table 1): 61 pregnant women (representing 62 pregnancies) and 68 controls. The majority of both pregnant women and controls were Africans (71% and 64.7%, respectively), the other ethnic groups being Caucasians (22.6% and 30.8%), Hispanic-Americans (4.8% and 0) and Asians (1.6% and 1.5%).
In the pregnant women group, the median age at delivery was 33 (range 22-47) years. All women gave birth to a living baby except one who experienced a miscarriage on week 9 of pregnancy. The mode of delivery was mostly elective caesarean section (69%), and 61% of newborns were boys.

Table 1: Characteristics of the study population

	Pregnant women group (n=61)				Control group (n=68)	
	Inclusion		Delivery			
Pregnancies[1]	62	(100%)	61	(98%)		
Miscarriage			1	[2]		
Age (years)	32.4	(22.2-46.7)	32.9	(22.3-46.9)	37.6	(23.9-64.7)
Ethnicity						
Caucasian	14	(22.6%)	13	(21.3%)	21	(30.8%)
Black	44	(71%)	44	(72.1%)	44	(64.7%)
Hispano-American	3	(4.8%)	3	(5%)	0	
Asian	1	(1.6%)	1	(1.6%)	2	(3%)
Unknown	0		0		1	(1.5%)
Body weight (kg)	69	(46-122)	75	(47-127)	62	(43-105)
Viral load (copies/mL)	<40	(<40-86000)	<40	(<40-422)	<40	(<40-33000)
CD4 cell count (cells/μL)	420	(54-1477)	455	(119-1477)	504	(42-1346)
Creatinine (μmol/L)	51	(29-81)	55	(24-100)	65	(28-135)
Smoke (cigarette/day)	0	(0-30)	0	(0-30)	0	(0-20)
Alcohol (dose/day)	0	(0-3)	0	(0-2)	0	(0-6)
ARV regimen studied	62	(100%)	61	(100%)	68	(100%)
LPV/r	40	(64.5%)	39	(64%)	36	(53%)
NVP	9	(14.5%)	9	(14.7%)	11	(16.2%)
ATV/r	5	(8.1%)	4	(6.6%)	15	(22%)
LPV/r, SQV	2	(3.2%)	2	(3.3%)	0	
ATV/r, SQV	2	(3.2%)	2	(3.3%)	0	
ATV	2	(3.2%)	2	(3.3%)	3	(4.4%)
LPV/r, NVP	1	(1.6%)	1	(1.6%)	1	(1.5%)
SQV/r	1	(1.6%)	1	(1.6%)	0	
NFV	0		1	(1.6%)	2	(2.9%)
Comedication (ARV)						
3TC	48	(77%)	48	(79%)	35	(51%)
ZDV	34	(55%)	35	(57%)	30	(44%)
TDF	14	(23%)	13	(21%)	31	(46%)
FTC	8	(13%)	7	(11%)	21	(31%)
ABC	11	(18%)	11	(18%)	6	(9%)
ddI	2	(3%)	2	(3%)	6	(9%)
d4T	1	(1.6%)	1	(1.6%)	1	(1%)
ENF	1	(1.6%)	1	(1.6%)	0	
Mode of delivery (n=60)[3]						
Caesarean section			41	(69%)		
Vaginal delivery			18	(31%)		
Gestational age (weeks)			37.1	(27.0-40.7)		
Infants[3]						
Boys			37	(61%)		
Girls			22	(36%)		
Infant body weight (g)			2930	(1350-3950)		
Infant HIV status						
3 months			61	negative		
6 months			61	negative		

Data are expressed as values (%), or as medians (range). [1] Data from pregnant women are reported to total number of pregnancies. [2] One miscarriage at first trimester (week 9). [3] Two loss of follow-up (no data).

The average weight gain at delivery was 7.5 kg, as compared with the postpartum period (taken as baseline).The mean postpartum body weight (BW) did not differ from the mean BW of controls (68.6 kg vs 65.2 kg, p>0.2).

4.1.3.2. Safety

All pregnant women responded well to ARV therapy. Their median HIV viral load at delivery was <40 copies/ml (range up to 422 copies/ml). None of the infants were infected with HIV (all tested negative at 3 and 6 months).

One woman experienced miscarriage on week 9, 6 (10%) suffered from pre-eclampsia, and 2 (3%) developed gestational diabetes. Other adverse events reported during the study were nausea (7%) and vomiting (5%). At birth, anaemia was reported in 8 (13%) newborns and malformations (without precision) in one.

4.1.3.3. Free and total plasma concentrations

Total and free plasma concentrations and free fractions of lopinavir (LPV), atazanavir (ATV) and nevirapine (NVP) observed in pregnant women and in controls are presented in Table 2 and Figure 1.

A slight, statistically non-significant decrease in LPV trough concentration ($C_{min,tot}$) of 11% was observed during the course of pregnancy (Figure 1a), while total LPV exposure (AUC_{tot}) decreased by 9% in the 3rd trimester (T3) (85.7 μg*h/ml) in comparison with postpartum (94.1 μg*h/ml). Pregnancy did not influence total LPV clearance (CL), distribution volume (Vd) or unbound fraction (f_u); neither were the extrapolated LPV free through concentrations ($C_{min,free}$) and free exposure (AUC_{free}) significantly different as compared to postpartum values. However, LPV f_u values were found slightly higher in samples taken during early pregnancy (1st trimester, T1), and steadily decreased over the trimesters until delivery (p=0.008) (Figure 1b). LPV pharmacokinetic parameters determined in the pregnant women group at postpartum did not differ from the control group of non-pregnant women.

Table 2: Pharmacokinetic parameters and protein binding for lopinavir (LPV), atazanavir (ATV) and nevirapine (NVP) in pregnant and control women.

	LPV							ATV							NVP						
	T1 (n=13)	T2 (n=33)	T3 (n=44)	Deliv (n=25)	Post (n=33)	Cord (n=25)	Controls (n=34)	T1 (n=3)	T2 (n=8)	T3 (n=7)	Deliv (n=6)	Post (n=7)	Cord (n=3)	Controls (n=16)	T1 (n=3)	T2 (n=7)	T3 (n=11)	Deliv (n=5)	Post (n=6)	Cord (n=5)	Controls (n=12)
Total parameters																					
C_{tot} (ng/ml)	6125 *	5994 *	6119	6029 *	7893	592	7945	1164	669 *	874	1035	1298	168	1368	5720	6087	5815	6706	7205	5738	7085
CV	32%	44%	46%	60%	50%	89%	44%	33%	78%	109%	84%	86%	59%	95%	37%	64%	56%	85%	53%	74%	43%
AUC_{tot} (ng*h/ml)	85752 *	83350 *	85724	86936	94149	NA	98968	40802	28956 **	29766 *	32294	40105	NA	38730	121155	100838	96078	107938	109395	NA	122084
CV	13%	20%	18%	21%	26%	NA	35%	8%	31%	51%	31%	29%	NA	23%	67%	49%	54%	51%	42%	NA	48%
$C_{min,tot}$ (ng/ml)	5823 *	5633	5751	5650	6397	NA	6489	639	291 **	546 *	513	707	NA	593	4257	4834	4733	5865	5958	NA	5185
CV	20%	26%	22%	33%	34%	NA	32%	25%	77%	70%	67%	76%	NA	57%	60%	72%	56%	85%	58%	NA	31%
CL (l/h)	4.7	5.0	4.8	4.8	4.5	NA	4.5	7.4	10.8 *	10.8	9.4	8.7	NA	8.9	3.2	3.2	3.2 **	3.0 *	2.7	NA	2.9
CV	14%	22%	20%	21%	25%	NA	21%	8%	32%	43%	27%	44%	NA	40%	36%	36%	34%	44%	40%	NA	44%
Vd (l)	72.2	72.4	71.8	68.3	71.1	NA	72.5	86.4	73.3 *	81.7	83.2	84.4	NA	83.8	110.6	96.6	103.0	104.2 **	103.8	NA	94.4
CV	9%	7%	6%	15%	12%	NA	6%	3%	17%	16%	10%	8%	NA	12%	42%	35%	27%	19%	38%	NA	23%
Free parameters																					
C_{free} (ng/ml)	58.9	54.3	55.2	51.2	62.8	12.1	52.2	105.6	58.7 *	81.6	96.0	119.2	33.3	108.7	2108.7	2227.0	2021.2	2066.2	2405.2	2172.4	3175.5
CV	36%	51%	76%	74%	74%	108%	45%	52%	71%	108%	79%	89%	64%	84%	20%	69%	56%	68%	45%	79%	26%
AUC_{free} (ng*h/ml)	816	747	741	707	709	NA	647	3707	2730 *	2760	2942	3794	NA	3201	44340	36351	33696	36123	37957	NA	55304
CV	28%	37%	51%	45%	52%	NA	30%	35%	28%	58%	28%	45%	NA	24%	53%	48%	50%	47%	38%	NA	47%
$C_{min,free}$ (ng/ml)	55.6	50.4	50.0	46.7	47.9	NA	42.7	59.7	25.2 **	50.0	50.2	66.9	NA	47.9	1520.1	1780.8	1635.5	1804.7	1981.4	NA	2343.8
CV	30%	39%	54%	52%	53%	NA	31%	49%	75%	71%	77%	94%	NA	53%	44%	77%	56%	70%	51%	NA	34%
F_u (%)	0.97 *	0.90 **	0.84 *	0.80	0.75	1.84	0.68	9.0	9.8	9.0	9.3	9.4	19.1	8.4	38.5	36.2	35.4	34.1	35.7	37.2	45.7
CV	34%	36%	38%	36%	46%	51%	25%	28%	27%	19%	14%	29%	10%	19%	19%	7%	16%	20%	21%	16%	16%
Protein concentrations																					
AAG (g/L)	0.63 ***	0.45 ***	0.46 ***	0.47 ***	0.74	0.15	0.66	0.72	0.60 **	0.65 *	0.61 **	0.92	0.22	0.79	0.77	0.59 **	0.52 **	0.56 **	0.83	0.21	0.80
CV	22%	34%	37%	41%	36%	112%	30%	43%	32%	37%	23%	22%	131%	30%	20%	31%	44%	49%	30%	76%	30%
HSA (g/L)	35.3 ***	33.3 ***	33.4 ***	31.9 ***	40.0	33.3	39.2	41.5	35.9 **	34.4 ***	31.7 ***	41.3	34.0	39.3	39.6 *	36.4 ***	35.5 ***	32.6 ***	45.3	37.1	42.6
CV	10%	11%	10%	14%	10%	9%	8%	11%	12%	11%	10%	11%	8%	10%	13%	5%	8%	17%	8%	10%	7%

Data are expressed as means and their associated coefficient of variation (CV). AAG, α-1-acid glycoprotein; AUC_{free}, free area under the time-concentration curve; AUC_{tot}, total area under the time-concentration curve; C_{free}, free observed concentration; $C_{min,free}$, free minimum concentration; $C_{min,tot}$, total minimum concentration; C_{tot}, total observed concentration; CL, apparent clearance; Cord, cord blood; CV, coefficient of variation; f_u, free fraction; HSA, human serum albumin; Post, postpartum; T1, trimester 1; T2, trimester 2; T3, trimester 3; Vd, distribution volume.

*P<0.05; ** P <0.01; *** P <0.001: Within-group comparison with postpartum.

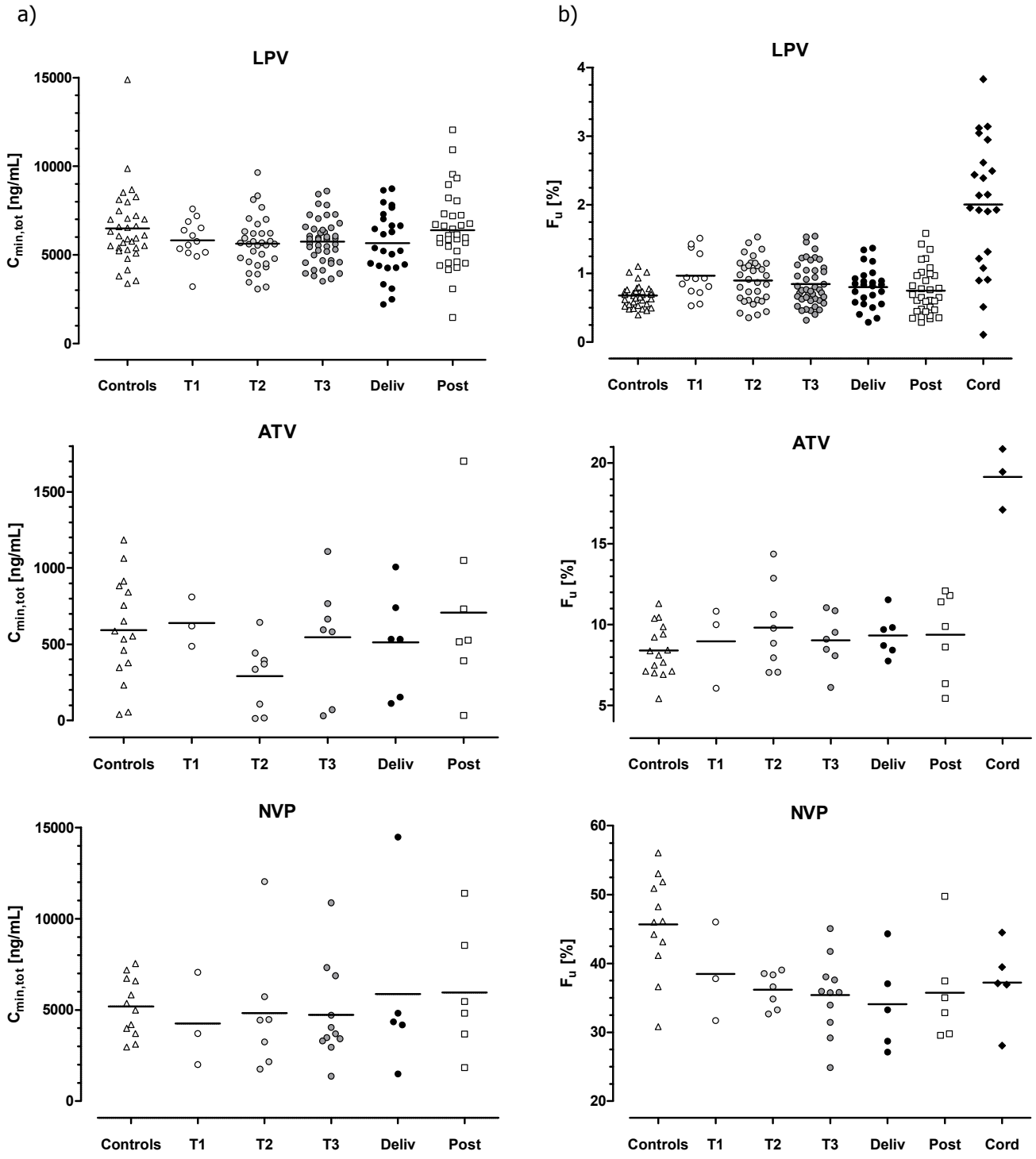

Figure 1: a) Total minimum concentrations ($C_{min,tot}$) derived from individual PK parameters and b) free fractions (f_u) for lopinavir (LPV), atazanavir (ATV) and nevirapine (NVP) according to stage of pregnancy versus controls. Full lines represent means. T1, trimester 1; T2, trimester 2; T3, trimester 3; Deliv, delivery; Post, postpartum.

Derived ATV $C_{min,tot}$ were also significantly lowered by pregnancy (p=0.026), mostly due to the 59% reduction at T2 (p=0.001) (Figure 1a), while total ATV exposure (AUC_{tot}) was found to decreased (p=0.019), principally during the 2nd trimester (T2) and T3 (27% reduction, p<0.05). When T2 values – clearly departing from other trimesters – were excluded from the statistical analysis, neither $C_{min,tot}$ nor AUC_{tot} remained statistically influenced by pregnancy (p=0.22 and 0.29, respectively). Analysis of variance for trimester indicated no statistically significant change induced by pregnancy for ATV $C_{min,free}$ and AUC_{free} (p>0.06). Comparison of ATV AUC_{free} determined at various trimesters vs. baseline (postpartum) values showed a 27% reduction at T2 and T3. Likewise, ATV $C_{min,free}$ was significantly lower at T2 (62% reduction, p=0.005) but by only 25% at T3 (p=0.08). The overall consequence was an unchanged ATV f_u during pregnancy (p=0.8) (Figure 1b). Furthermore, ATV CL and Vd variations were not statistically significant between trimesters (p>0.08), although, again at T2, CL appeared slightly increased and Vd slightly decreased (p<0.05) as compared to postpartum values. All ATV PK parameters were similar between controls and pregnant women at postpartum.

For NVP, a non-significant 22% reduction was found for the extrapolated $C_{min,tot}$ (p>0.2) (Figure 1a). We also observed a 10%, non-significant reduction in NVP AUC_{tot} at T2 and T3. Overall, pregnancy appeared to have negligible influence of NVP free exposure: average NVP $C_{min,free}$ decreased by 16% during pregnancy, whereas NVP AUC_{free} was reduced by 4% and 11% at T2 and T3, respectively, compared to postpartum (NS). Accordingly, pregnancy seemed not to impact on NVP f_u (Figure 1b), whereas CL increased to some extent (by 20% at T3, p=0.002) by comparison with postpartum. Of note, NVP f_u determined in the control group was significantly different from f_u observed in the pregnant group at postpartum (p=0.015).

4.1.3.4. Placental transfer

Among the 30 maternal-cord paired samples collected, placental transfer for total and free concentrations could be assessed in 16 for LPV, 3 for ATV and 3 for NVP. Eight paired samples (6 LPV and 2 NVP) with questionable or unstated time collection were discarded. Mean (CV) cord-to-mother (C/M) ratios for total concentrations were 0.16 (85%), 0.18 (87%) and 0.92 (25%) for LPV, ATV and NVP, respectively. C/M ratios for free concentrations were 0.43 (83%), 0.32 (82%) and 1.07 (14%) for LPV, ATV and NVP, respectively.

4.1.3.5. Protein binding

Both AAG and HSA concentrations decreased significantly during pregnancy compared to postpartum (Table 2 and Figure 2). AAG levels were 15% lower already during T1 ($p=0.001$), and reached 34% reduction at delivery, compared to postpartum ($p<0.0001$). HSA levels were also affected from early pregnancy (9% lower, $p<0.0001$), and decreased by 23% at delivery ($p<0.0001$). Postpartum protein concentrations were not statistically different from those in the control group ($p>0.2$).

Umbilical cord blood exhibited significantly lower AAG concentrations than maternal blood at delivery (average 0.16 g/L vs 0.51 g/L, $p<0.0001$), whereas HSA concentrations were essentially similar (34.0 g/L and 31.9 g/L, respectively, $p=0.04$). Accordingly, f_u of LPV and ATV measured in cord plasma were approximately 2-fold higher than those measured in mother plasma (1.84% vs 0.80%, and 19.1% vs 9.3%, respectively, $p<0.0001$), whereas NVP f_u was not significantly different between umbilical cord and mother plasma (37.2 % vs 34.1%, NS).

4.1.3.6. Correlation between AAG, HSA, total and free concentrations

Figure 3 shows the regression between total plasma concentrations (C_{tot}) and free plasma concentrations (C_{free}) for each drug, with coefficient of linear correlation (r) of 0.86, 0.95 and 0.98 for LVP, ATV and NVP, respectively.

For LPV and ATV, the regression improved on introducing both AAG and HSA concentrations in the simplified model (eq. 2). The inclusion of a saturable protein binding component did not further improve the model for both drugs. Thus, LPV and ATV f_u increased as a result of AAG and HSA levels decreasing during pregnancy. For NVP, the C_{free}-C_{tot} regression was not explained to any relevant extent by AAG and HSA concentrations. Thus, there is no indication that protein modifications induced by pregnancy influence NVP free fraction.

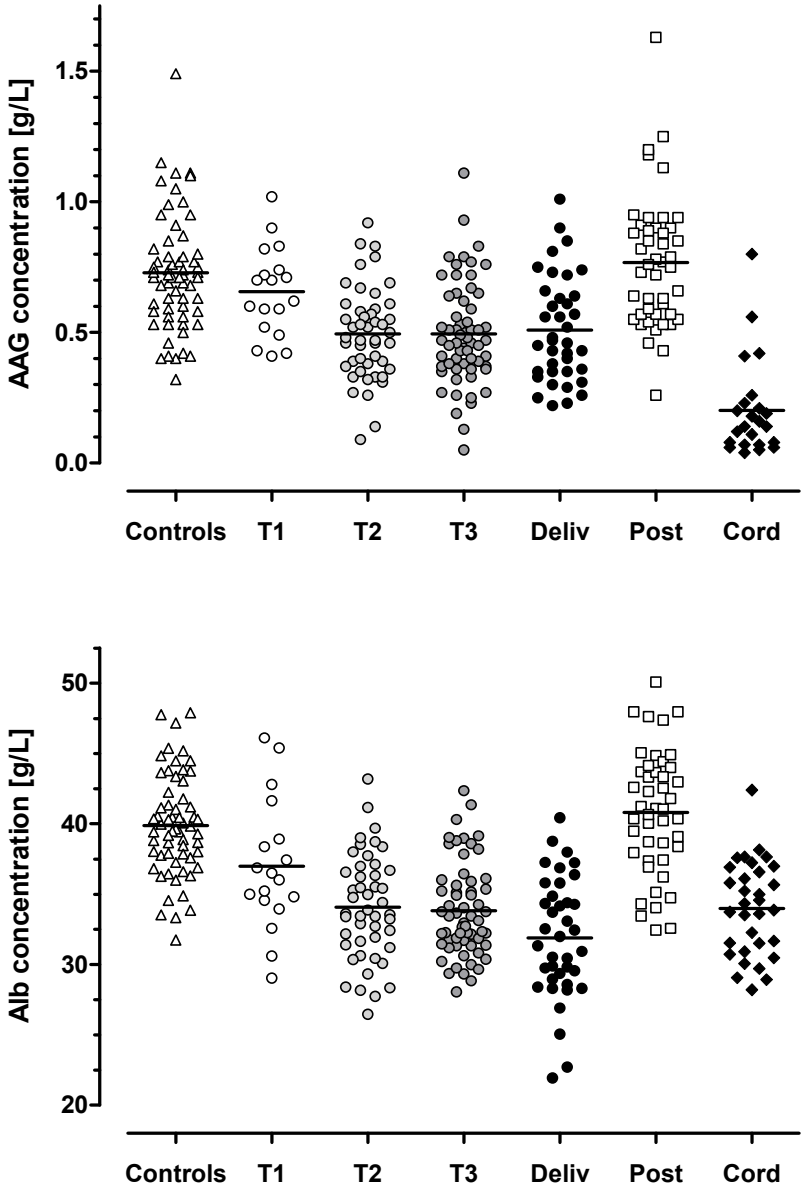

Figure 2: Influence of pregnancy on α-1-acid glycoprotein (AAG) and human serum albumin (HSA) levels.

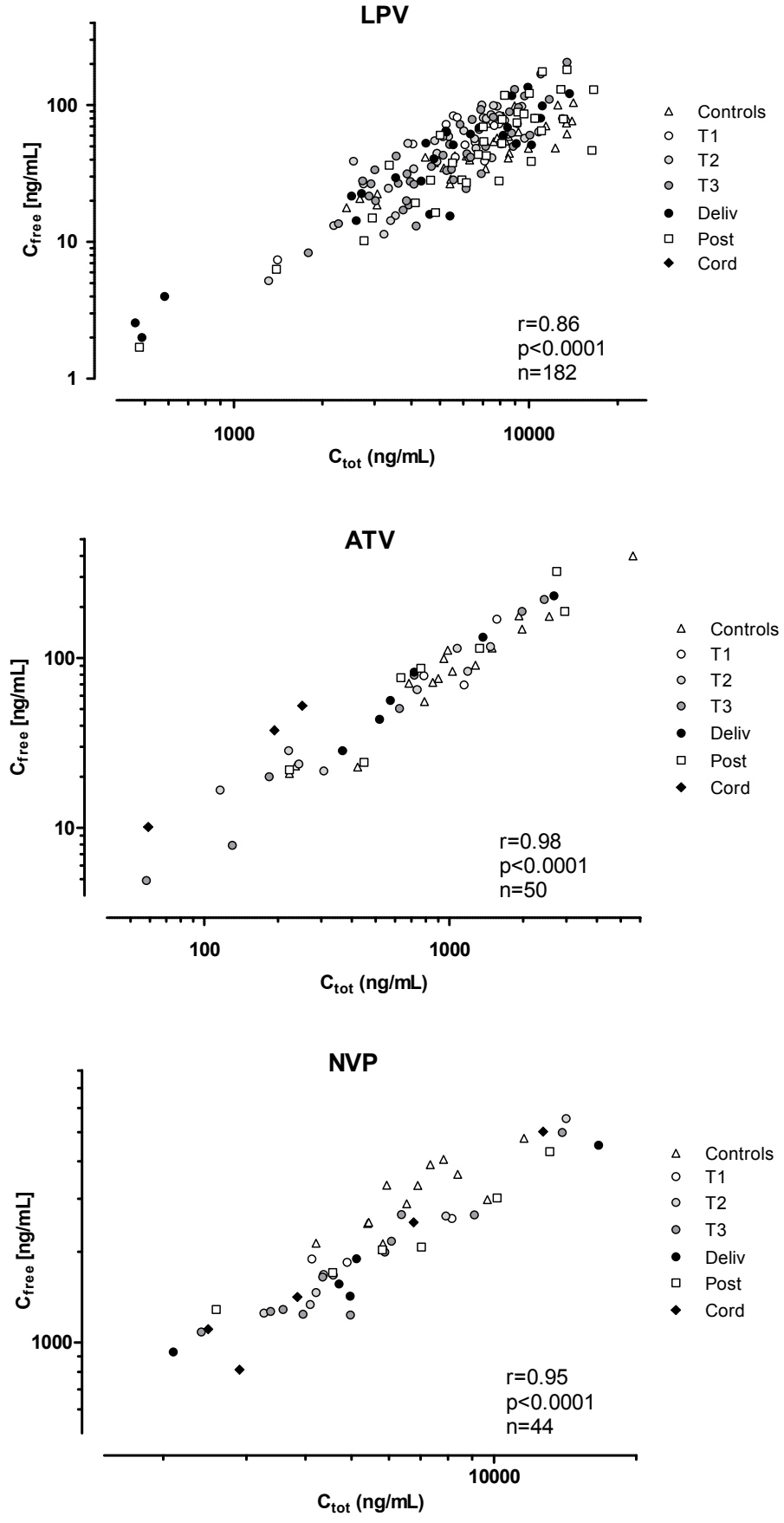

Figure 3: Free plasma concentrations (C_{free}) *versus* total plasma concentrations (C_{tot}) for lopinavir (LPV), atazanavir (ATV) and nevirapine (NVP)

4.1.4. Discussion

Pregnant women are generally excluded from clinical trials, and the impact of physiological changes inherent to their specific condition on the disposition of drugs is poorly known or based on pathophysiological considerations only. In the current DHHS "Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents" (11), the use of Therapeutic Drug Monitoring during pregnancy is advocated but its role in clinical practice is poorly defined. The possibility of drug dosage modification in pregnant women is evoked, but the rationale onto which drug adjustment should be based in not clear. Thus, the clinical significance of possible changes in exposure during pregnancy remains at present unclear for most ARVs.

To the best of our knowledge, this is the first study having followed both the total and free – pharmacologically active – plasma concentrations of LPV, ATV and NVP in pregnant women during the course of pregnancy, at delivery, and in postpartum. A few reports on total antiretroviral drug exposure during pregnancy have been published or presented in abstract form (22-41). Prospective studies following patients over the entire course of pregnancy are difficult to perform, and pharmacokinetic studies published so far have been generally based on a limited number of patients followed for a single antiretroviral drug. Results are frequently conflicting, reflecting the variability of the pregnant women population, the diversity of study designs and possibly analytical limitations.

In our population of 42 pregnant women who completed the study under LPV-based regimen, we found no significant effect of pregnancy on total LPV exposure (AUC_{tot}). Accordingly, derived LPV $C_{min,tot}$ remained unaltered. Overall, average total LPV exposure was 10% lower during pregnancy, a decrease unlikely to be of clinical relevance. Importantly in our study, the extrapolated LPV $C_{min,tot}$ remained in all pregnant women above the target trough concentration (C_{trough}) of 1000 ng/ml recommended against wild-type HIV-1 viruses (11). Moreover, only 11% of LPV $C_{min,tot}$ values measured in patients were below 4000 ng/ml, the suggested minimum target trough plasma concentration for treatment-experienced patients (42).

Previous studies on the pharmacokinetics of total LPV exposure during pregnancy reveal discrepant and sometimes conflicting results. In a population of 17 HIV-infected pregnant women, LPV C_{min} were reported to be reduced by 56% in T3 compared to postpartum, with only 18% (3/17) of the pregnant women reaching the 10^{th} percentile (P10) exposure of 52 μg*h/ml found in non-pregnant patients (22). In our study, only 2

of the 148 AUC_{tot} were below this target, and this was possibly due to incorrect times reported for last drug intake. In another study presented in abstract form, Peytavin *et al*. (23) found 26% and 36% reductions in total LPV C_{min} at T2 and T3, respectively, in 101 HIV-infected pregnant women compared to a control non-pregnant group. Other published studies reported lower LPV C_{min} during T3 or at delivery than ours, however without comparison with a postpartum period or a non-pregnant population (24-26). By contrast, Baroncelli *et al.* (27) found C_{trough} of 5500 and 5300 ng/ml for respectively treatment-experienced and naïve pregnant women during T3, results in close agreement with our data. In two studies from the Pediatric AIDS Clinical Trials Group (PACTG) Protocol P1026s (43), adequate LPV PK at T3 were reported with administration of an increased LPV dose of 600mg BID (tablets) (28) or 533mg BID (soft gel capsules) (29). However, in the latter study (29), a significant 19% of pregnant women under the increased dose still did not reach the target AUC of 52 μg*h/ml. Alternately, maintenance on the high dosage regimen until 2 weeks postpartum resulted in very high LPV exposures among patients. In fact, the decision to increase LPV dosage in this study was based on the earlier report by Stek *et al*., in which a 56% reduction in C_{min} during T3 was reported in 17 pregnant women (22). However, the consequence of increased LPV dosage on free concentrations, the only species likely to cross placental barrier, was not addressed in those studies.

We observed no impact of pregnancy on free LPV exposure (AUC_{free} and $C_{min,free}$) in our study. LPV f_u was highest at T1 and remained generally higher during pregnancy (+29% and +13% at T1 and T3, respectively) comparatively to postpartum. Aweeka *et al*. (30) also reported an 18% increase in f_u at T3 compared to postpartum in 29 pregnant women, although with a different methodology. These results are consistent with LPV being a highly protein-bound drug with a low hepatic extraction ratio (<0.3); a change in protein binding induced by pregnancy thus induces only a transient increase in free drug concentration, so that more free drug is available for metabolic clearance. Therefore, even though total LPV exposure is apparently slightly reduced, free LPV AUC remains unaffected. Our results are consistent with theoretical pharmacokinetic considerations, and suggest that dosage adjustment is not required for LPV during pregnancy. Most pregnant women (37/42; 88%) on LPV were virologically suppressed prior to delivery under a standard LPV 400mg BID dose in our study, and no MTCT was observed.

Means ATV C_{tot} and C_{free} during T2 were especially low compared to all other periods combined. Antiretroviral exposure with boosted or unboosted ATV-based regimen is

more vulnerable to a reduced adherence, which was not formally assessed during our study. Ritonavir, used as booster for ATV – and also monitored with our assay – was not detected in some samples from T2, suggesting either poor compliance to this agent or drug interactions with unreported comedications (proton pumps inhibitors) influencing ATV biodisposition or increased gastric pH due to pregnancy. These reasons and the limitation of the small sample size and large inter-individual PK variability of ATV have been acknowledged by Ripamonti *et al*, in a study of 17 pregnant women with low total ATV exposure in pregnancy compared to postpartum (31).

Regardless of T2 values, pregnancy seemed to have a comparable influence on free and total PK, with both free and total exposures reduced by *ca* 23-28% in late pregnancy. Similar results for total exposure were found in a study of 12 pregnant women, with ATV AUC and C_{min} reduced by 40% and 21% respectively, compared to historical data (32). However, reported C_{min} were all above the suggested minimum C_{trough} for wild-type HIV-1 virus of 150 ng/ml (11). In our study, most (77%) ATV $C_{min,tot}$ in pregnant women were also >150 ng/ml. Of note, ATV was given to patients either boosted or unboosted: in all but one samples with ATV $C_{min,tot}$ below 150 ng/ml, RTV was not detected. Finally, one pregnant woman reported not taking RTV, because of gastric intolerance. Mean ATV $C_{min,tot}$ measured in our pregnant women population at T3 was 513 ng/ml, in agreement with the values (619 ng/ml and 421 ng/ml) reported in two other studies determined at the same period (33,34), with also a few values below the recommended C_{trough}.

Up to now, conflicting results have been published regarding total NVP pharmacokinetics in pregnancy. In the PACTG 1022 trial, NVP AUC_{tot} was reported to be similar between T2, T3 and postpartum in 12 pregnant women (35). Similar results were found in a population of 26 pregnant women, with NVP AUC_{tot} of 56 μg*h/ml antepartum and 61 μg*h/ml postpartum (36). By contrast, von Hentig *et al*. found an increased NVP clearance and low total exposure in 16 pregnant women, compared to a non-pregnant population (37). Similarly, NVP PK was significantly altered in a population of 20 pregnant women, none of them reaching C_{trough} of 4000 ng/ml at delivery (24). In a more recent study, only 55.6% of the pregnant women achieved adequate C_{trough} (3100 ng/ml) in late pregnancy (27).

Our study did not find any significant influence of pregnancy on either total or free NVP exposure, compared to postpartum, with f_u remaining unchanged as well. The regression between C_{tot} and C_{free} was not affected by AAG and HSA. NVP being a drug with low hepatic extraction and moderate protein binding (approximately 60%), the increase in total clearance in response to changes in protein levels during pregnancy

was not expected to reflect into a significant alteration in free exposure. A modification in NVP dosage is therefore not required during pregnancy.

In line with previous reports, total placental transfer (C/M ratios) found in our study were particularly low for LPV (22,28,29,38) and ATV (31,34,39,40), whereas NVP crossed the placenta more extensively (38,40,41). This is probably the consequence of PIs, unlike NVP, being substrates of P-glycoprotein, an efflux drug transporter highly expressed in placenta (44). With our sensitive LC-MS/MS technology, we were able to detect not only total but also free concentrations of LPV, ATV and NVP in most of our umbilical cord plasma samples. Interestingly, we observed that free C/M ratios were 1.8 to 2.7-fold higher than total C/M for PIs. This is partly accounted for by the difference in protein concentration between maternal and fetal plasma. Indeed, a transplacental AAG level gradient favors partition of total drug on the maternal side for highly protein-bound drugs like PIs. In our study, AAG concentration in umbilical cord blood was markedly lower than in maternal blood, therefore the free fraction of LPV and ATV, mainly bound to AAG (98% and 86% respectively), was higher in cord blood than in maternal blood. Thus, free C/M ratios values suggest that the fetus may be exposed to higher free PIs concentrations than predicted from total C/M ratios. This phenomenon was not observed for NVP, given comparable HSA levels in maternal and fetal plasma.
In conclusion, total PK changes, but most importantly free PK changes observed during pregnancy were either barely detectable for LPV or modest for ATV and NVP. Such small variations in PK during pregnancy are unlikely to have clinical consequences. This conclusion is strengthened by the fact that no MTCT were observed during the study. Our findings do not call for systematic adjustments of LPV, ATV and NVP dosage during pregnancy.

4.1.5. Methods

4.1.5.1. Study design

This prospective, multicentre, observational study was conducted within the framework of the Swiss HIV Cohort Study (SHCS) (45) and Mother and Child HIV Cohort Study (MoCHIV). Five academic Swiss HIV clinics (Geneva, Lausanne, Zurich, Bern and Basel) participated. Blood samples were collected during pregnancy in the first trimester (T1, i.e. until week 14), the second trimester (T2, i.e. between week 15 and 26), the third trimester (T3, i.e. from week 27 to the end of pregnancy), on delivery (one sample from

the mother, and one from the umbilical cord blood, if possible at the same time), and one or two months postpartum. The samples were collected at any time after last dose intake, but the exact dose intake and sampling times were recorded into a case report form (CRF), along with information on dosing regimen, demographic data, body weight (BW), hypertension, co-medication, pregnancy-related adverse events (nausea, vomiting,…), viral load, CD4 cell count, HSA, AAG, creatinine, ASAT, ALAT and blood count.

4.1.5.2. Patients

All HIV-infected women announcing a pregnancy and treated with at least one PI and/or NVP were offered participation in the study. In addition, a corresponding group of non-pregnant HIV-infected women under PIs or NVP was enrolled as control. Since the vast majority of pregnant women were on a LPV-, ATV- or NVP-based regimens, only these three ARVs were considered in the analysis. The study protocol was approved by the corresponding institutional ethic committees. Written informed consent was obtained before participation from all patients.

4.1.5.3. Drug measurements

Total plasma concentrations (C_{tot}) of ATV, LPV and NVP were measured by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after protein precipitation with acetonitrile, using an adaptation of our previously reported method (46). The limits of quantification (LOQs) for this assay were 50 ng/mL for ATV, 100 ng/mL for LPV, and 250 ng/mL for NVP. Our laboratory participates in an international external quality assurance program for the analysis of concentrations of antiretroviral drugs (KKGT, Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie, Association for Quality Assessment in TDM and clinical Toxicology, The Hague, The Netherlands). In the last 3 external quality control rounds, the deviations from target QC values were always comprised within -6 to 0%, -7 to 6%, and -9 to 5% for LPV, ATV and NVP respectively.

Ultrafiltration was used to separate the free fraction from the total plasma concentration using a methodology developed and validated in our laboratory (47). Free plasma concentrations (C_{free}) were then determined in batch by LC-MS/MS. For each analyzed drug, the LOQ for the assay was 1 ng/mL.

4.1.5.4. Human serum albumin and α-1-acid glycoprotein determinations

HSA and AAG concentrations were measured using commercially available assays from Roche Diagnostics based on colorimetric and immunoturbidimetric methods, respectively, carried out on a Roche Cobas Integra 400 apparatus (Roche Diagnostics, Rotkreuz, Switzerland).

4.1.5.5. Placental transfer

Total and free drug placental transfer was evaluated by the ratio of the respective total and free concentration in umbilical cord plasma over maternal plasma at delivery (C/M ratio). Whenever possible, maternal and cord bloods were sampled at the same time, but never more than 6 hours apart (paired samples taken more than 6 hours apart were not included). When total and free drug concentrations in cord were below the limit of quantification (LOQ) of our assay, they were equated by default to half the LOQ values (this occurred in 4 out of 30 paired samples).

4.1.5.6. Pharmacokinetics analysis

Total plasma concentrations were extrapolated to trough ($C_{min,tot}$) in each individual patient through Bayesian calculations using the observed random sample concentration, sampling and dosage time, assuming maximum likelihood values of individual apparent clearance (CL) and distribution volume (Vd), referred to their average value and variability found in population pharmacokinetic descriptions (48-49). The total area under the concentration-time curve (AUC_{tot}) over a dosing interval was estimated by dividing the administered daily dose by CL. Free drug fraction (f_u) was calculated as the ratio of C_{free} over C_{tot}. The free area under the concentration-time curve (AUC_{free}), and minimum concentration ($C_{min,free}$) were then extrapolated from their total counterparts multiplied by f_u.

4.1.5.7. Statistical analysis

The influence of the pregnancy stage (T1, T2, T3, delivery and postpartum) on total and free drug exposures (AUCs and C_{min}), f_u, CL, Vd, AAG, HSA and BW was assessed by analysis of variance. The relationships between C_{tot} and C_{free} and between f_u and AAG or HSA were explored by standard linear regression analysis. The combined influence of AAG and HSA levels on the ratio of C_{tot} to C_{free} was examined by nonlinear regression. According to the law of mass action, C_{tot} can be expressed as follows:

$$C_{tot} = \frac{C_{free} \times L_{AAG} \times AAG}{C_{free} + K_{dAAG}} + \frac{C_{free} \times L_{HSA} \times HSA}{C_{free} + K_{dHSA}} \quad (1)$$

where L_{AAG} and L_{HSA} are constants composed of both molecular weight ratios and number of binding sites per molecule of AAG and HSA, respectively, and K_{dAAG} and K_{dHSA} are the respective dissociation constants describing drug affinity to *AAG* and *HSA*.

A simplified linear model was also tested, taking AAG and HSA first separately, and then together (eq. 2):

$$C_{tot} = C_{free} \times (F_{AAG} \times AAG + F_{HSA} \times HSA) \quad (2)$$

where F_{AAG} and F_{HSA} are composite coefficient factors for AAG and HSA, respectively, which integrate L/K_d ratios.

Alternative models were explored as well (e.g. saturable binding to AAG with linear binding to HSA, etc.). Statistical significance was assumed at a *P*-value less than 0.05.

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4.2. Population Pharmacokinetics of Lopinavir in HIV-infected pregnant women

In the study on free and total plasma concentrations of ARVs during pregnancy presented previously (Section 4.1.), we have obtained answers to the questions of clinicians whom asked themselves about the necessity to adapt or not antiretroviral drug dose in pregnant women.
Now, with the wealth of data obtained for lopinavir essentially in this study, we have initiated a more formal population pharmacokinetic (PopPK) analysis using the NONMEM program (see. §.2.5.), to better understand the influence of pregnancy and different covariates on the pharmacokinetics of lopinavir.

At present, this PopPK analysis is still ongoing, and the first results obtained so far will be presented now.

4.2.1. Introduction

HAART is now the standard of care to prevent mother-to-child HIV transmission. Moreover, lopinavir/ritonavir (LPV/r) in combination with lamivudine/zidovudine is the treatment of choice for pregnant women (1).
LPV/r is characterized by a large pharmacokinetic (PK) inter-individual variability in the general population (2,3). Part of this variability may be explained by body weight, age sex, α-1-acid glycoprotein plasma levels, drug-drug interactions, liver disease, poor adherence or host genetic factors. Moreover, pregnancy may also explain part of this variability (3-8).

It is essential for pregnant women to achieve at least relevant trough concentrations to maintain viral load below the limit of detection (<40 copies/ml) to prevent mother-to-child transmission of HIV. However, as already discussed, pregnancy is known to be associated with physiological changes that may result in significant modifications of the PK of many drugs. Although some published studies have demonstrated a decrease in LPV exposure during pregnancy and an increase in LPV dosage have been proposed by some authors, we have found in our study described in Section 3.1. that pregnancy did

not influence significantly LPV exposure, and thus do not call for a systematic dosage adjustment of LPV during pregnancy.

Because the exposure of LPV depends on various factors influencing PK variability, a population pharmacokinetic (PopPK) analysis would be interesting to better understand this variability.

To date, only one PopPK of LPV in pregnant women have been published (9), and have demonstrated that pregnancy status, along with gestational age, significantly influence LPV clearance. Other PopPK xx in non-pregnant population found significant influence of other covariates, such as body weight, presence of a CYP3A4 inducer (efavirenz) or the CYP3A4 inhibitor ritonavir, α-1-acid glycoprotein levels, and genetic polymorphisms, on either clearance or volume of distribution or both (10-13).

The objective of this population study is to investigate total LPV PK in pregnant women using a more formal population approach than those used previously (§ 4.1.). The addition in the model of available data on free LPV concentrations is expected to improve the population model.

4.2.2. Material and Methods

4.2.2.1. Patient population

All HIV-infected women announcing a pregnancy and treated with at least one PI and/or nevirapine (NVP) were offered to take part in the study. In addition, a corresponding group of non-pregnant HIV-infected women under PIs or NVP was enrolled as control. For this formal population pharmacokinetic analysis, were have kept only pregnant women and controls receiving lopinavir.

Blood samples were collected during pregnancy during first, second and third trimesters, on delivery, and one or two months postpartum. The samples were collected at any time after last dose intake, but the exact times of intake and of sampling were recorded into a case report form (CRF), along with information on dosing regimen, demographic data, body weight (BW), hypertension, co-medication, pregnancy-related adverse events (nausea, vomiting,…), viral load, CD4 cell count, HSA, AAG, creatinine, ASAT, ALAT and blood count.

4.2.2.2. Analytical method

Blood samples were collected according to the method described in Section 3.1.3 in the frame the previous study on ARVs in pregnancy. Total plasma concentrations (C_{tot}) of lopinavir (LPV) were measured by LC-MS/MS after protein precipitation with acetonitrile, using an adaptation of our previously reported method (14). Ultrafiltration was used to separate the free fraction from the total plasma concentration using the methodology developed and validated in our laboratory and described elsewhere (15). Free plasma concentrations (C_{free}) were then determined in batch by LC-MS/MS.

4.2.2.3. Model-based pharmacokinetic analyses

The analysis was performed using the NONMEM® computer program written in FORTRAN 77 (version VI, with NM-TRAN version II) (16). It uses mixed (fixed and random) effects regression to estimate population means and variances of the pharmacokinetic parameters and to identify factors that influence them.

4.2.2.4. Structural model

A stepwise procedure was used to find the model that fitted the data at best: first, 1- and 2-compartment models with zero- and first-order absorption from the gastrointestinal tract were compared based on the average value and variability found in population pharmacokinetic descriptions in the literature (9,10,13). The estimated parameters were systemic clearance (CL), volume of distribution of the central compartment (V_d) and absorption rate constant (ka). Since LPV was only administered orally, CL and V_d represent apparent values (CL/F, respectively V_d/F, where F is the oral bioavailability).

4.2.2.5. Statistical model

Exponential errors following a log-normal distribution were assumed for the description of inter-patient variability of the pharmacokinetic parameters and were of the form shown below:

$$\theta_j = \theta \cdot e^{\eta_j}$$

where θ_j is the individual pharmacokinetic parameter value in the j^{th} individual, θ the population parameter estimate and η_j the random effect value, which is independently and normally distributed with a mean of zero and variance Ω.

Proportional, additive and combined proportional-and-additive error models were compared to describe the intra-patient (residual) variability.

4.2.2.6. Covariate model

The covariate analysis will be performed using a stepwise approach. Visual inspection of the correlation between post-hoc individual parameter estimates and the available covariates will be first conducted by graphical exploration. Potentially influential covariates will be then incorporated sequentially into the pharmacokinetic model.

In brief, the typical value of a given parameter θ (e.g., CL) is modeled to depend either linearly on the covariate X : $\theta = \theta_a(1 + \theta_b \cdot X)$, or as a power function: $\theta = \theta_a \cdot X^{\theta_b}$, where θ_a is the average estimate and θ_b is the relation deviation (positive or negative) of the average attributed to the covariate X.

Baseline covariates (X) evaluated for inclusion during the model building are body weight, age, ethnicity, trimester of pregnancy, gestational age, α-1-acid glycoprotein and albumin levels. At the end of the analysis, all patients characteristics that showed an influence on the parameters will be evaluated again by comparison of the full model (with all factors included) with a model with each of the factors will be deleted sequentially.

4.2.2.7. Parameter estimation and model selection

The models were fitted by use of the first-order conditional method (FOCE in NONMEM) with the subroutine ADVAN 2, TRANS 2. Goodness-of-fit statistics and graphical displays were used to compare models on each step of model building. The goodness-of-fit criterion was the change in the objective function (OF) resulting from the addition of one covariate, which approximates a χ^2 distribution and can be regarded as statistically significant ($p < 0.05$) if it exceeds 3.8 for one additional parameter.

A simulation based on the final pharmacokinetic estimates will be performed with NONMEM® using 1,000 individuals to calculate 95% prediction intervals of the concentrations vs. time curve.

4.2.3. Preliminary results

4.2.3.1. Demographic data

A total of 184 LPV observations from 41 pregnant women (representing 42 pregnancies) were included in the population analysis: 12 observations for the first trimester (T1), 34 for the second trimester (T2), 44 for the third trimester (T3), 27 observations on delivery, and 33 observations in postpartum (considered as baseline). In addition, a total of 34 LPV observations were collected from 33 non-pregnant women (control group).

All patients received lopinavir/ritonavir 400/100 mg twice daily except one control who received 800/200 mg once daily, always in association with other antiretroviral agents. The characteristics of the population studied have already been depicted in Table 1 of Section 4.1.

4.2.3.2. Population pharmacokinetic analysis

A one-compartment model with first-order absorption for the gastrointestinal tract was found to describe appropriately the dataset. Neither a two-compartment model nor a one-compartment with zero-order absorption further reduced the objective function.

The assignment of an interindividual variability term on CL and V_d improved the fit (ΔOF= –26 and –5.3, respectively). Allowing for interindividual variability on the absorption rate constant k_a improved the fit as well, but its estimate was very high owing to a limited number of data during the absorption phase (ΔOF= –8.5). Since the absorption rate constant could not be estimated appropriately, k_a was fixed to 0.3 h^{-1} based on the average value found in population pharmacokinetic descriptions in the literature (9,10,13).

The use of a proportional error model for the residual intra-patient variability was the most satisfactory. A combined proportional-and-additive error model did not improve the model significantly (ΔOF= –2.2).

The pharmacokinetic estimates and the variabilities (CV) of the population model without covariate were CL = 4.66 L/h (28%), V_d = 50.6 L (75%) and k_a = 0.3 h^{-1}. The diagnostic plots generated for this intermediate model are shown in Figure 1.

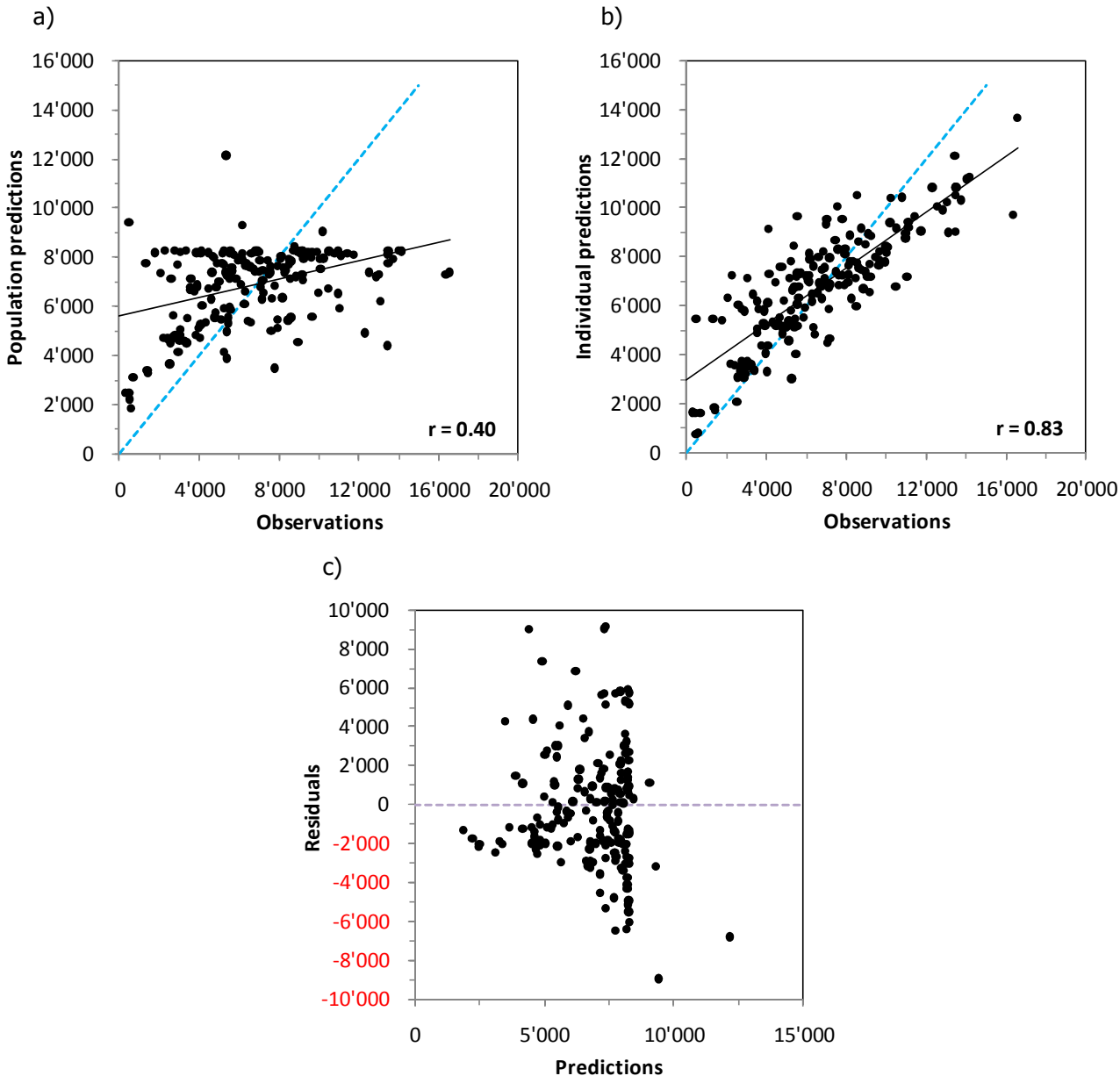

Figure 1: Diagnostic plots of the population model without covariate, all concentration data are given in ng/ml: a) observed *values* versus population prediction values, the dotted line is the line of identity and r is the correlation coefficient; b) observed values *versus* individual predicted values, the dotted line is the line of identity and r is the correlation coefficient; c) population prediction *versus* population residual values, the dotted line is at ordinate value zero.

The model with covariates is ongoing. Preliminary data showed that addition of the body weight (BW) on CL and V_d did not significantly improve the pharmacokinetic model (ΔOF= –2.7 and –1.7, respectively).

Further data of the impact of other covariates (age, ethnicity, trimester of pregnancy, gestational age, α-1-acid glycoprotein and albumin levels) on CL and V_d will be soon available.

4.2.4. Discussion

These preliminary data of our population PK model in pregnant and non-pregnant women is reassuring, as the same initial model without covariates has been already found for a population of non-pregnant adults and recently published by our group (13).

Only one article on population PK of LPV in HIV-infected pregnant women has been published to date (9). Our actual pharmacokinetic estimates of the interim population analysis are in agreement with data found in this population PK (9), where pharmacokinetic estimates and variabilities (CV) of the initial model were: CL = 5.07 L/h (31%), V_d = 58.8 L (59%) and k_a = 0.255 h^{-1}. But contrary to our findings, body weight was shown to influence CL in the population of this latter study (9).

When our LPV population PK will be completed, we should be able to confirm the results found in the clinical study and presented earlier in Section 3.1. We should thus expect from our population PK model that the addition of pregnancy as covariate has no impact on LPV CL and V_d, whereas addition of α-1-acid glycoprotein and albumin might improve the fit. Of importance, a significant influence of α-1-acid glycoprotein on LPV CL has already been reported in a population PK model in HIV-infected non pregnant adults (6). By contrast, Bouillon-Pichault *et al.* (9) found in their population PK study in pregnant women a significant influence on LPV CL of pregnancy status (cut-off of 15 weeks (gestational age) to separate pregnant and non-pregnant women), as well as of gestational age when combined with pregnancy status.

Since LPV is characterized by a large pharmacokinetic inter-individual variability in the general population, it is not impossible to that we will find results not in accordance with previously published data. As previously mentioned, it is thus expected, with this population PK, to confirm our previously findings, which do not call for a systematic dosage adjustment of LPV during pregnancy. Finally, this will be the first time that free concentrations of LPV will be included in a popPK in HIV-infected pregnant women.

4.2.5. References

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4.3. Placental transfer of antiretroviral drugs

The human placenta is a unique organ whose structure is different from that of other animal species in that human placental barrier is composed of a single rate-limiting layer of cells called *syncytiotrophoblasts*. Besides its role of transferring nutriments and oxygen to the foetus and of removing waste products to the mother, the placenta offers a protective barrier for the developing foetus by reducing the entry of various xenobiotics from the mother to the foetus, while for others it facilitates their passage both to and from the foetal compartment. Since placental transfer of drugs from the maternal to the foetal side occurs primarily via passive diffusion, the physicochemical properties of drugs such as lipid solubility, polarity and molecular weight primarily determine the rate of transfer across the placenta. The degree of plasma protein binding may also affect the amount transferred. Of importance, only the non-protein-bound fraction of a drug is free to diffuse by passive transfer across the placenta (1,2).

Besides passive diffusion, placental transfer may involve active transport by transmembrane protein "pumps" requiring energy. As shown in Figure 1, a wide variety of uptake and efflux transporters are expressed in the placenta, at the maternal-facing brush border (apical) or the foetal-facing basolateral (basal) membranes of the syncytiotrophoblast (2).

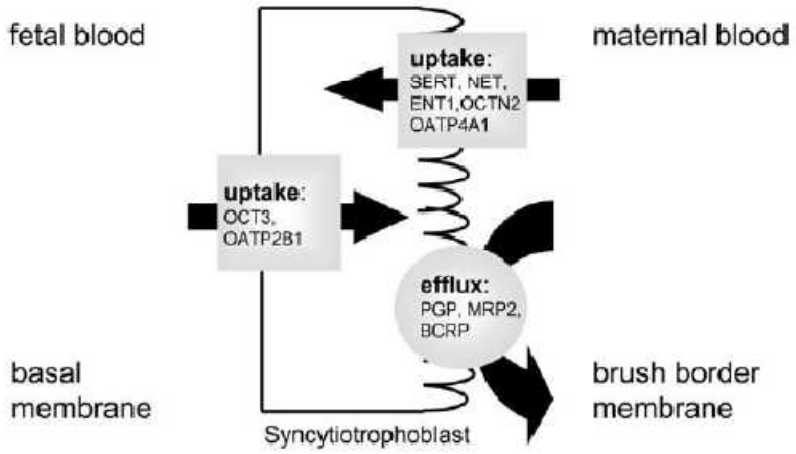

Figure 1: Schematic representation of syncytiotrophoblast depicting localisation of uptake and efflux transporters. SERT, Serotonin transporter; NET, norepinephrine transporter; ENT1, equilibrative nucleoside transporter; OCTN2, organic cation/carnitine transporter; OATP4A1, organic anion transporting polypeptide 4A1; OCT3, organic cation transporter 3; OATP2B1, organic anion transporting polypeptide 2B1; PGP, P-glycoprotein; MRP2, multidrug resistance protein 2; BCRP, breast cancer resistance protein. *Taken from*: Marzolini et al. Placental transfer of ARV drugs. Clin Pharmacol Ther 2005;78(2):118-122.

Depending on their function and localisation, they facilitate the transport or the removal of drugs between the foetus and the maternal circulation.

Among the transporters thought to be of importance to drug disposition, P-glycoprotein (P-gp) is one of the most extensively studied and best characterised efflux transporters in the placenta (3). P-gp is present on the brush border membrane of the syncytiotrophoblast, where it limits intracellular accumulation of xenobiotics. Of relevance to foetal drug exposure, polymorphisms in *MDR1* seem to be correlated with placental P-gp expression (4,5).

Since HAART has become part of standard care for HIV-infected pregnant women during pregnancy, foetus may be exposed to ARV drugs. PIs, which are substrate of P-gp, do not cross the placenta to an appreciable extent and are, therefore, unlikely to provide any direct antiviral protection or toxicity for the newborn (6-11). By contrast, efficient passage of NNRTIs and NRTIs across the placenta has been reported (6,8,12-14), and may thus protect foetus against HIV infection.

As previously discussed (see § 4.1.), several publications on pharmacokinetics of ARVs during pregnancy have recommended to increase in ARVs drug doses during late pregnancy reportedly to ensure that pregnant women will have sufficient drug levels for full viral suppression (9,15-19). However, during our study on placental transfer (see § 3.1.) we have shown that the free fractions of LPV and ATV in the foetal circulation (umbilical cord blood) were more than 2-fold higher than free fractions in the maternal circulation. Thus, the foetus appears to be exposed to higher free concentrations than expected on the basis of placental transfer of total plasma levels, the only species in maternal blood considered up to now. Whether an increase in free concentrations consequently to a dose modification may potentially affect the foetus has never been assessed. This is however an important issue in terms of foetal toxicology warranting further studies on the pharmacokinetics of free ARV drugs and on the impact on dose modification on foetal exposure (20).

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Chapter 5:
Optimising ART in special populations

In another field of investigation aiming at optimizing ART therapy in HIV patients, we have focused on a population comprising individuals with repeatedly high plasma concentrations of antiretroviral drugs, principally of efavirenz. We have studied the possibility to adjust their dosage downward using TDM with possible consequences on treatment tolerability, patients convenience and treatment economicity. The efavirenz dosage adjustments guided by TDM were also compared to those based on pharmacogenetics markers.

Efavirenz, in combination with tenofovir/emtricitabine, is the preferred NNRTI for treatment naïve patients. Its long half-life (40-55 h) allows a once daily administration with a low pill burden. EFV-based regimens have demonstrated virological superiority compared to some PIs-based regimens, including lopinavir/ritonavir combination. Despite this, its major limitation is its central nervous system (CNS) adverse effects, which were demonstrated in most, but not all, studies to be correlated with high EFV plasma levels. Such adverse drug reactions appear to occur especially at the treatment initiation and seem to attenuate thereafter, but these side effects may remain very uncomfortable for some patients under EFV treatment, thereby enhancing the risk of treatment interruption or poor adherence. Thus, any strategy likely to increase treatment tolerance is sought to prevent both treatment failure and severe CNS toxicity.

The following study (§ 5.1.) on EFV have focused on the usefulness of therapeutic drug monitoring to guide dosage adaptation in patients with high EFV plasma concentrations.

Both second and third articles presented thereafter (§ 5.2. and § 5.3.) also deal with EFV therapy and are to be considered as two companion papers as both pharmacogenetics and pharmacokinetics results herein are nurturing themselves reciprocally. These studies have enabled a better understanding of the main and accessory metabolic pathways of EFV using a comprehensive pharmacogenetic-pharmacokinetic approach, a method offering the best appreciation of the multi-dimensional aspects of HIV pharmacotherapy.

The analytical method for the measurement of EFV and its metabolites – described in Section 2.5. – has been developed purposely for these studies for being able to establish the detailed EFV metabolite profiles of patients carefully characterized by their EFV pharmacokinetics and by relevant pharmacogenetics markers.

5.1. Successful efavirenz dose reduction led by Therapeutic Drug Monitoring

Efavirenz (EFV) being metabolized mainly by cytochrome P450 (CYP) 2B6, less by CYP3A4 and 2A6, plasma EFV concentrations in patients is potentially influenced by polymorphisms of these genes, known to have functional consequences. Thus, some patients may experience high plasma levels of EFV often associated with side effects or toxicity, thereby increasing the likelihood of treatment discontinuation.
Several studies have demonstrated the relationship between EFV plasma concentration and clinical efficacy and toxicity. Thus, EFV dose reduction in patients with high plasma concentrations has been evaluated and seems generally to decrease the rate of treatment discontinuation and side effects, without compromising clinical efficacy, while leading to treatment cost reduction for patients.

Even though a significant number of patient followed at the HIV unit at CHUV have already had their EFV dosage regimen adjusted by clinicians in accordance to plasma levels, there is only few controlled trial evaluating the safety and clinical benefit of reduced dosage regimens of EFV guided by TDM.
In that context, a clinical study has been initiated in collaboration with the HIV Units at CHUV (Lausanne) and at HUG (Geneva), which aims to assess the role of Therapeutic Drug Monitoring for dose adjustment in patients with plasma EFV concentration above percentile 75, and genotype for CYP2B6, CYP3A4 and CYP2A6 relevant variants. This study also aimed at ascertaining whether systematic genetic testing may either replace or complement TDM to guide EFV-based therapy.

Both HIV units were in charge of the recruitment of patients on stable EFV regimen, while the Division of Clinical Pharmacology at CHUV was in charge of drug analysis by liquid chromatography coupled to tandem mass spectrometry, clinical interpretation and dose adjustment proposition, using a simplified algorithm based on Bayesian pharmacokinetic approach.

Related Appendices: 5.1, 5.2

SUCCESSFUL EFAVIRENZ DOSE REDUCTION LED BY THERAPEUTIC DRUG MONITORING

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Antiviral Therapy 2010 *(in press)*

5.1.1. Abstract

5.1.1.1. Background

There are potential benefits to adjusting dosage in patients treated with efavirenz (EFV). We tested a simplified algorithm based on a Bayesian pharmacokinetic approach for guiding dose reduction in patients with EFV concentrations above the 75th percentile (P75) with documented virological efficacy.

5.1.1.2. Methods

We designed a prospective, open-label, multicenter study. All consenting participants with EFV concentrations above P75 on standard dosage were included in a dose-reduction cycle. Primary endpoint was the number of patients who reached plasma concentrations within targets (1000-4000 ng/mL) after at most two cycles of dose reduction at 3 and 6 months. *CYP2B6* genetic characterization was performed and adherence monitored electronically.

5.1.1.3. Results

Seventy-two patients were screened and 13 fulfilled selection criteria. These patients, with undetectable viremia on a stable 600 mg-EFV-based regimen, had a median (IQR) EFV plasma level of 8'112 ng/mL (5'993-10'278) at baseline; 38% were between P75 and P95, qualifying for a 400 mg-EFV dose, and 62% above P95, qualifying for a 200 mg-EFV dose. After one to two dose-reduction cycles, all patients reached targets for EFV plasma concentration (P25-P75) at 24 weeks. The predictive dose reduction based on genetic profile differed from dose reduction according to therapeutic drug monitoring (TDM) in 3 patients. All patients maintained viral suppression at 6 months.

5.1.1.4. Conclusion

A standardized TDM-guided EFV dose-reduction strategy over a 24-week period was successful and safe and yielded EFV plasma concentrations within the recommended therapeutic range. In addition to the improvement in several neuropsychiatric parameters, EFV dose reduction based on plasma concentration has the potential to substantially decrease treatment cost.

5.1.2. Introduction

Therapeutic drug monitoring (TDM) of efavirenz (EFV) identifies high or low concentrations which may be responsible for increased toxicity or for decreased efficacy [1]. In April 2003, the first version of a guideline to optimize TDM in HIV clinical care was presented. An update has been published in 2006 and recommends using TDM in selected patients, such as those with impaired renal or hepatic function, pregnancy, or suspected drug-drug interaction, for example [2].

Investigations regarding the benefit of TDM in unselected populations have yielded conflicting results [3-6], owing to the lack of standardised criteria in measurement of ARV plasma concentration, or in defining thresholds for dose change. A recent Cochrane systematic review concluded that there is a lack of support for routine use of TDM, but also underscored the lack of data to identify selected populations that may benefit from its use [7].

Prospective studies have demonstrated the relationship between EFV plasma concentration and clinical efficacy and toxicity even in long-term users [1,8-10]. Moreover, EFV is metabolized mainly by cytochrome P450 (CYP) 2B6, and its concentration is associated with *CYP2B6* variation [11]. A recent study has tested the feasibility of a *CYP2B6* genotype-based dose reduction of EFV, with less CNS symptoms in 10 out of 14 patients who received a reduced dosage [12]. More recently, the result of the ATHENA cohort demonstrated that TDM-guided EFV dose reduction in patients with high plasma concentrations did not compromise virological efficacy and led to a decreased risk of treatment discontinuation [13]. Beside benefits in terms of tolerability, dose reduction can also impact on individual treatment cost. Minimum effective dose can vary with inter-individual differences in body weight, pharmacogenetic make up and other factors [14]. Dose recommendations have often been derived from clinical trials that were not designed to establish minimum effective doses for individual patients. For example, EFV has been licensed by US FDA in 1998 at a fixed 600 mg QD dose. A phase II trial (DMP 005) showed no difference in efficacy between 200, 400 and 600 mg QD doses, when combined with AZT and 3TC [15-16].

We aimed at testing a simplified algorithm for dose reduction in patients with documented virological efficacy, treated by a stable EFV 600 mg QD based regimen while presenting with elevated plasma concentration. We used a standardised algorithm, based on a Bayesian population pharmacokinetic model developed by our group [17], to reduce doses in patients with plasma EFV concentration above percentile 75 (P75). We hypothesized that dosage individualisation was feasible and safe.

5.1.3. Materials and methods

5.1.3.1. Patients

Patients were recruited at two University Hospitals (Lausanne University Hospital CHUV, Service of Infectious Diseases, and Geneva University Hospital, HIV Unit, Switzerland) from November 2006 to March 2008. All patients were on stable EFV regimen (600 mg QD), with a viral load below 40 copies for at least 3 months. An EFV plasma concentration found above P75 at screening was confirmed on baseline, and the patients had to sign an informed consent form.

The protocol was approved by the Human Research Ethics Committees of Lausanne and Geneva University Hospitals. The study was conducted in accordance with the ethical principles laid out in the Declaration of Helsinki (1996) and Good Clinical Practice guidelines (Consolidated guidelines [E6] issued by the International Conference on Harmonization [ICH] in May 1996).

5.1.3.2. Study design

This study was a prospective open-label multicenter trial on patients screened with EFV plasma concentration above P75, measured between 8 to 24 hours post-dose. Blood samples were taken at least 8h after last EFV dose intake, to ensure that sampling was taken during the elimination phase of the drug. All patients fitting with inclusion criteria underwent a dose reduction according to a standardized scheme based on a Bayesian approach (Figure 1). This approach evaluates the most likely contribution of intra- and inter-individual variability in EFV kinetics, and adjusts the dosage specifically with respect to inter-individual variability. Drug dosage was modified according to the protocol on week 2, after reception of the baseline confirmation of EFV plasma concentration above P75. Patients with EFV concentration between P75 and P95 received EFV 400 mg QD (two 200 mg tablets), while those with concentration above P95 received EFV 200 mg QD (one tablet). Plasma concentrations were then checked again on week 6. If EFV concentration was still above targets, a second cycle of dose reduction was to be performed. EFV plasma concentrations were then monitored on weeks 10 and 24 for all patients. The protocol did not allow for more than 2 cycles of drug dosage adjustment. The primary endpoint was the number of patients who reached a plasma concentration within targets (1000-4000 ng/mL) after at least one cycle (and maximum two cycles) of dose reduction at 6 months, according to the

provided algorithm. The percentage of patients remaining with undetectable viral load at 3 and 6 months was also determined as a secondary endpoint.

Other laboratory measurements were also performed on baseline, and on weeks 10 and 24: viral load, CD4 cell count, serum ALT, total cholesterol, triglycerides, HDL, LDL, glucose, creatinine, full blood cell count, and pregnancy test (if required).

All patients underwent questionnaires on baseline, week 10 and week 24. All questionnaires were administered by a trained study nurse. Symptoms of depression, anxiety and stress were assessed with the Depression Anxiety and Stress Scale (DASS) [18]. This scale was chosen for its high internal consistency, temporal stability and stable factor structure [19-20]. The DASS scale distinguishes among normal, mild, moderate, severe and extremely severe degrees of depression, anxiety or stress. Sleep quality was measured using Groningen Sleep Quality Score (GSQS) [20].

Finally, patients were genetically characterized for cytochrome P450 (*CYP*) *2B6*, *2A6* and *3A4* [21], in order to document the impact of polymorphisms on EFV concentrations and prediction of dose reduction (see below).

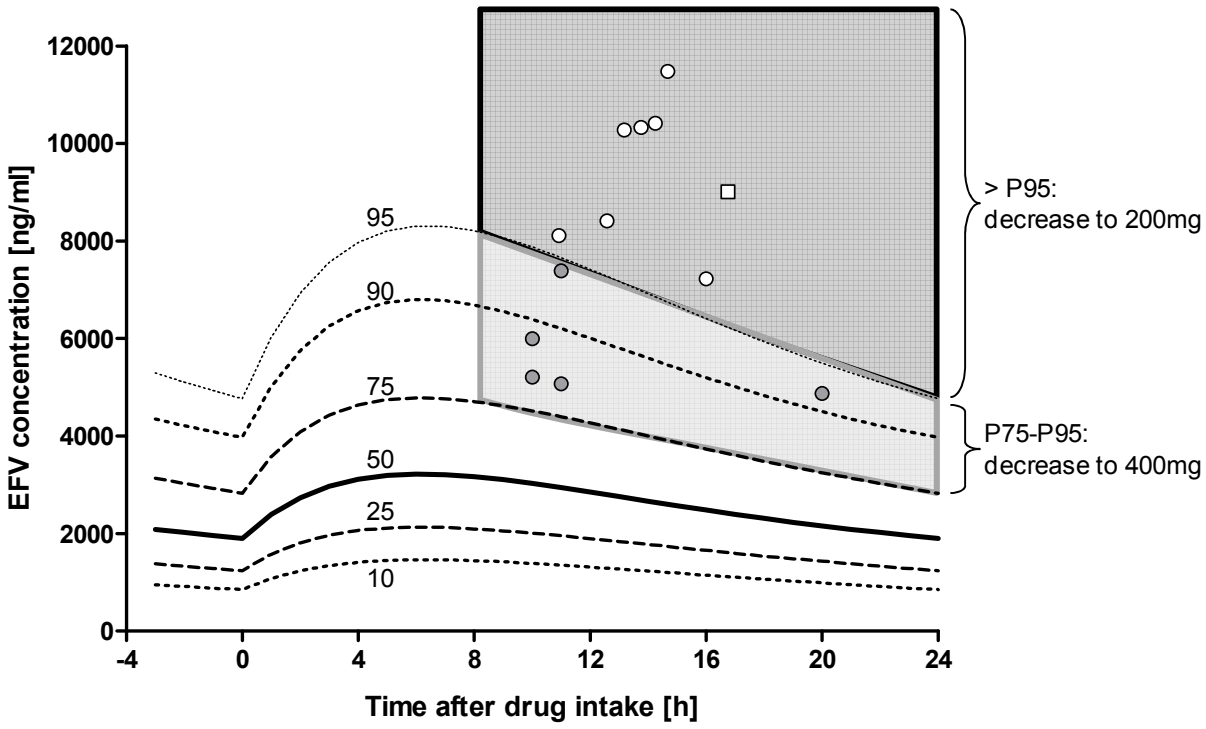

Figure 1: Percentile curves for efavirenz (EFV) dose adjustment based on a Bayesian approach, for patients receiving 600 mg/day.
Circles and squares represent EFV plasma concentrations at baseline for patients included in the study (n=13). White circles: patients above percentile 95, qualifying for a 200 mg dose; White square: patient above percentile 95 who needed 2 cycles of dose reduction; Grey circles: patients between percentiles 75 and 95, qualifying for a 400 mg dose

5.1.3.3. Plasma concentration

EFV total plasma concentrations were determined by high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS/MS) after protein precipitation with acetonitrile (MeCN) using an adaptation of our previously reported methods [22-23].

EFV pure substance, kindly provided by MSD (Merck Sharp & Dohme-Chibret AG, Glattbrugg, Switzerland), was used to prepare calibration and quality control samples. The internal standard (I.S.) used was efavirenz-d_4 (EFV-d_4), kindly provided by TRC (Toronto Research Chemicals Inc., North York, Canada).

We used selected transitions and collision energy previously reported [22], with some minor modifications: the *m/z* transition, collision energy (V) and tube lens used are 314.0 → 244.0, 29 and 60 for EFV, and 318.0 → 248.0, 27 and 37 for EFV-d_4. The range of calibration curves was established up to 10'000 ng/ml, with a lower limit of quantification of 250 ng/ml. The laboratory participates to an international external quality assurance program for the analysis of concentrations of antiretroviral drugs (KKGT, *Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie*, Association for Quality Assessment in TDM and clinical Toxicology, The Hague, The Netherlands). In the last 3 external quality control rounds, the deviations from target QC values were always comprised within 1 to 6%.

5.1.3.4. Adherence

Adherence to EFV was assessed by using an electronic pill-container, the Medication Event Monitoring System (MEMS®, AARDEX) [24], which allowed us to assess longitudinally the patient's drug dosing history (i.e. date and exact time of each opening of the pill-container), and to compare it with the prescribed drug dosing regimen and drug plasma level. Electronic data were reconciliated with pill count and patients' interview. EFV adherence was defined as the percentage of days with correct dosing according to physician's prescription.

5.1.3.5. Genetic analysis

CYP2B6 genetic characterization was carried out for all patients. When CYP2B6 function was impaired, additional characterization of the accessory pathways CYP2A6 and 3A4 was performed [11, 25-27].

In order to comprehensively characterize CYP2B6 function in patients with plasma concentrations above P75, all nine *CYP2B6* exons and intron-exons boundaries (4299bp)

were fully resequenced. Primers and conditions were previously published [11, 28]. On account of the working hypothesis that *CYP2A6* and *CYP3A4* loss-of-function alleles would be clinically relevant among patients with impaired CYP2B6 metabolism [21, 27], *CYP2A6* and *CYP3A4* were characterized in patients homozygous for a loss-of-function (LOF) allele of *CYP2B6.* Given the large number of functional alleles associated with CYP2A6 decrease/loss-of-function and their high prevalence, *CYP2A6* characterization included full resequencing (promoter region, all nine exons and intron–exons boundaries [4357 bp]), gene conversion and gene copy number assessment, as described [21]. Given the paucity of functional alleles associated with CYP3A4 decrease/loss-of-function despite extensive investigation by many groups over the years [29], only the two most frequent and potentially functional alleles/variants *CYP3A4*1B* [30-31] and *CYP3A4* rs4646437 [32] were genotyped by TaqMan allelic discrimination. Primers and probes were described elsewhere [33] or obtained by assay on demand at Applied Biosystems (TaqMan® SNP Genotyping Assay: C_32306227 10), respectively for *CYP3A4*1B* and *CYP3A4* rs4646437 genotyping. Genotyping results did not interfere with the decision process of lowering the EFV dosage and were submitted to a post–hoc analysis. However we hypothesized that all patients included in this study should be homozygous for a loss-of-function allele of *CYP2B6*, as they were chosen to undergo EFV dose reduction according to TDM data. In order to compare the predictive effectiveness of the genotype in comparison to TDM, we built the following prediction model: patients with impaired CYP2B6 metabolism, but no loss-of-function in the accessory pathways (CYP2A6 and CYP3A4), were expected to need a one unit EFV dose reduction (=400mg QD). Patients with impaired CYP2B6 metabolism and carrying one or more loss-of-function alleles in *CYP2A6* and/or *CYP3A4* were expected to need a two unit EFV dose reduction (= 200mg QD).

5.1.3.6. Statistical analysis

Differences in EFV plasma concentration, in scores for DASS questionnaire, and in safety parameters between screening and/or baseline visits and week 24 were analyzed with a non parametric Wilcoxon matched-pairs test with a significance threshold of 5%. The quantitative score for GSQS questionnaire was collapsed into a binary variable: undisturbed to moderately disturbed sleep (score≤5) versus strong disturbed sleep (score>5), and analyzed using a chi-square test with a threshold of 5%. Statistical analysis was performed using STATA Release 10.0 (Stata Statistical Software: Release 10.0, Stata Corporation, College Station, USA).

Table 1: Patients' baseline characteristics

	N (%)	Median (IQR)
Patients :	13 (100)	
from Geneva	7 (53.9)	
from Lausanne	6 (46.1)	
male	8 (61.5)	
age (years)		45 (35-50)
weight (kg)		68 (62-75)
height (cm)		171 (160-181)
HIV disease severity (CDC score) at treatment initiation :		
A	6 (46.2)	
B	2 (15.4)	
C	5 (38.4)	
HIV duration (months) :		79.7 (37.0-108.2)
Ethnicity (%)		
Caucasian	7 (53.8%)	
Black	4 (30.8%)	
Latin American	2 (15.4%)	
Drugs (median duration expressed in months) :		
efavirenz	13 (100)	51.2 (16.7-79.3)
abacavir	7 (53.8)	16.2 (5.7-71.2)
lamivudine	8 (61.5)	30.9 (10.2-62.0)
emtricitabine	5 (38.5)	18.3 (13.1-20.4)
tenofovir	6 (46.1)	20.8 (18.3-37.2)
EFV concentration (ng/ml) :	13 (100)	8'112 (5'993-10'278)
between P75 and P95	5 (38.5)	5'206 (5'076-5'993)
above P 95	8 (61.5)	9'644 (8'335-10'348)

IQR: interquartile range; P75: percentile 75; P95: percentile 95.

5.1.4. Results

5.1.4.1. Patients

Out of 72 subjects screened for the study, fifteen (21%) were candidates for EFV dosage adjustment at baseline. Two subjects decided to stop the study at baseline, and were excluded from the final analysis. One withdrew consent, the other one had incident tuberculosis. Thus, thirteen patients were included in the study at baseline (Table 1): 62% were male, median age was 45 years; 7 were Caucasian from Western Europe, 4 were born in Africa and 2 were from Hispanic/Latin origin. All had undetectable viremia,

and were exposed to EFV since 4.3 years (median) on baseline. Additionally, they were receiving either the combination of emtricitabine and tenofovir (n=5), or abacavir and lamivudine (n=7), or tenofovir plus 3TC (lamivudine) (n=1). Six patients were also on non-HIV medications such as anti-inflammatory drugs (ibuprofen, n=2), lipid-lowering drugs (n=2), ACE inhibitors (n=2) or neuropsychiatric drugs (escitalopram, benzodiazepine, n=1).

5.1.4.2. Dose adaptation

Screening and baseline samples were drawn between 9.25h and 22.3h after last dose intake. On baseline, five patients (38%) had EFV concentrations between P75 and P95, qualifying for a 400mg dose (Figure 1). As a result, the median (IQR) EFV plasma concentration decreased from 5'206 (5'076-5'993) ng/ml on baseline to 3'021 (2'937-3'261) ng/ml on week 6 (p=0.043). Eight patients (62%) had concentration above P95 and underwent a dose reduction directly from 600mg QD to 200mg QD (Figure 1). In this group, median (IQR) EFV concentrations decreased from 9'644 (8'335-10'348) ng/ml to 2'483 (2'111-2'861) ng/ml (p=0.012). For one patient, dose reduction was erroneously done at 400mg QD instead of 200mg QD on the first cycle, and required a second cycle of dose reduction to reach the recommended therapeutic interval.

After dose reduction, EFV drug concentrations remained above the trough threshold of 1'000 ng/ml recommended by FDA in all patients (Figure 2). Moreover, all EFV plasma concentrations (median, range) remained below 4'000 ng/ml on week 10 (2'826 ng/ml, 1'571-3'760) and on week 24 (2'707 ng/ml, 1'604-3'940).

Interestingly, the reduction in concentration after dosage adjustment was greater than the dosage reduction itself, both in the patients who changed to 400mg QD (41% versus 33%) and in those who changed to 200mg QD (76% versus 67%); this was not an effect of sampling time differences.

5.1.4.3. Virological status

At the beginning of the study, all patients had a viral load (VL) below 40 copies/mL by protocol. On weeks 12 and 24, all patients except one remained undetectable. One patient had an isolated blip on week 24 (54 copies/mL), but VL was again below the detection level (<40 copies) on subsequent monitoring 12 months after study interruption without dosage change. This patient had been adjusted to an EFV dose of 400 mg QD.

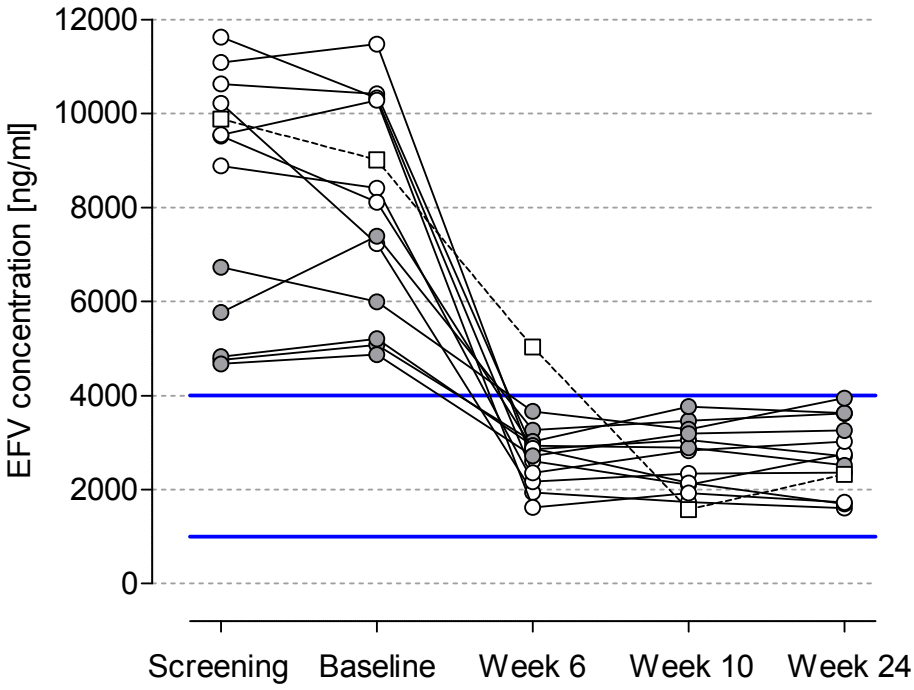

Figure 2: Efavirenz concentrations before and after dose reduction in patients (n=13) during the study. White circles: patients qualifying for a 200 mg dose; White square with dotted line: patient who needed 2 cycles of dose reduction; Grey circles: patients qualifying for a 400 mg dose.

5.1.4.4. Safety

We observed a significant reduction in anxiety scores (p=0.036) and a trend in lower stress scores (p=0.077) in patients after dose reduction on week 24, as measured by the DASS questionnaire (Table 2). We also recorded small but significant increase in CD4 cell count between study start and termination. By contrast, no change in sleep quality or length was identified. During the study period, one serious adverse event was declared; the patient experienced acute renal insufficiency and rhabdomyolysis that was thought to be related to cocaine consumption and not to the study drug.

5.1.4.5. Adherence

Eleven patients, out of the thirteen included, used the electronic pill-container. One patient refused the electronic pill-container and another one often prepared EFV doses in advance, thus invalidating the adherence data, which were discarded from the analysis. The median (IQR) adherence to EFV was 99% (98-100) in the 11 included subjects. In a total of 1771 monitored days, there were only 31 (1.7%) days without any EFV drug intake, whereas 15 (0.8%) days were reported with more than one EFV intake.

Table 2: Safety and questionnaires results at baseline and week 24

	Median (IQR) or N (%)		p value
	Baseline	Week 24	
DASS questionnaire :			
Stress score	4 (2-14)	0 (0-6)	0.077
Anxiety score	6 (2-10)	0 (0-4)	0.036
Depression score	2 (0-8)	0 (0-10)	0.788
Score GSQS :			0.125
Undisturbed to disturbed sleep (GSQS score≤5)	9 (69.2)	10 (76.9)	
Very disturbed sleep (GSQS score>5)	4 (30.8)	3 (23.1)	
Safety :			
ALAT	28 (23-39)	32 (21-44)	0.972
Total cholesterol	5.9 (5.3-6.5)	5.4 (5.2-6.4)	0.100
Triglycerides	1.2 (0.9-1.8)	1.4 (1.1-2.0)	0.208
HDL	1.8 (1.7-2.1)	1.7 (1.4-1.9)	0.162
LDL	3.2 (2.8-3.9)	3.1 (2.7-3.7)	0.402
Glucose	5.5 (5.1-6.1)	5.3 (5.0-5.7)	0.382
Creatinin	74 (64-80)	74 (63-82)	0.700
White blood cells	6.1 (5.1-7.2)	5.7 (5.1-7.3)	0.552
Hemoglobin	142 (123-150)	144 (124-148)	0.159
Platelets	214 (200-255)	219 (185-270)	0.196
CD4	513 (448-591)	570 (513-609)	0.023
HIV Viral Load	40 (40-40)	40 (20-40)	0.263

5.1.4.6. Genetic analysis

Twelve patients were characterized for *CYP2B6*, *2A6* and *3A4*. No genetic consent could be obtained for one patient. All patients were homozygous for a *CYP2B6* loss-of-function (LOF) allele, except one patient who was heterozygous for an uncharacterized *CYP2B6* allele. As we could not determine whether this allele led to a loss-of-function, we included this patient for the further assessment of the accessory pathways CYP2A6 and CYP3A4. Three patients did not carry any *CYP2A6* or *CYP3A4* LOF, and were therefore expected to need a one unit EFV dose reduction (=400 mg QD). Nine patients had at least one *CYP2A6* and/or *CYP3A4* LOF, and were therefore expected to need a two unit EFV dose reduction (=200mg QD). The results are shown in Table 3. When comparing the predictive EFV dose reduction according to either TDM or genotype, all but 3 patients were classified in the same category and would have received a similar EFV dosage.

Table 3: Effective and predictive dose reduction according to Therapeutic Drug Monitoring (TDM) and genotype respectively

Patient	Genotype			Dose reduction		Interpretation of EFV concentration (percentile)	
	CYP2B6	*CYP2A6*	*CYP3A4*	Effective (TDM)	Predictive (genotype)	Baseline	Week 24
1	2LOF	1LOF	0LOF	200 mg	200mg	> P95	P25
2	2LOF	1LOF	2LOF	200 mg	200mg	> P95	P25-P50
3[a]	2LOF	0LOF	2LOF	200 mg	200mg	> P95	P25-P50
4	2LOF	0LOF	0LOF	200 mg	400mg[b]	> P95	P25-P50
5	2LOF	1LOF	0LOF	200 mg	200mg	> P95	P50-P75
6	2LOF	0LOF	2LOF	200 mg	200mg	> P95	P25-P50
7	2LOF	1LOF	2LOF	200 mg	200mg	> P95	P50-P75
8	2LOF	2LOF	2LOF	200 mg	200mg	> P95	P50-P75
9	2LOF	0LOF	0LOF	400 mg	400mg	P75-P95	P50-P75
10	1 or 2 LOF	0LOF	0LOF	400 mg	400mg	P75-P95	P25-P50
11	2LOF	1LOF	0LOF	400 mg	200mg[b]	P75-P95	P50-P75
12	2LOF	0LOF	2LOF	400 mg	200mg[b]	P75-P95	P50-P75
13[c]	-	-	-	400 mg	-	P75-P95	P50-P75

a) patient who needed 2 cycles of dose reduction due to an error; b) predictive dose reduction according to genotype different from effective dose reduction according to TDM; c) patient not genotyped. LOF= loss/decreased of function.

5.1.5. Discussion

Our study indicates that TDM-guided EFV dose reduction is successful and safe, without any negative impact on virological outcome at follow up. The use of a standardized Bayesian algorithm enabled correct dose reduction to target therapeutic range (1000-4000 ng/ml), without reaching subtherapeutic concentrations. Adherence to EFV was monitored electronically in order to insure high level drug intake.

There are several reports suggesting that EFV could be used at reduced dose. Firstly, dose recommendations have often been derived from clinical trials that were not designed to establish minimum effective doses in individual patients. Phase II dose-ranging studies did not show differences in virological efficacy among 137 naïve patients randomized to 24 weeks of treatment with zidovudine and lamivudine plus efavirenz at doses of 200mg, 400mg or 600mg once daily, or matching placebo. There was no difference in HIV RNA suppression rates between the three doses of efavirenz and these efficacy results were sustained to week 24. [15-16]. Secondly, a study of 180 individuals reduced 49 patients with high plasma drug concentrations from 600 mg down to 400 mg without reporting virological risks with this strategy [13]. Our study shows that with a clear algorithm built up on population pharmacokinetic data to derive a simple percentile-based strategy, drug reduction is feasible without jeopardizing efficacy.

Reducing drug exposure has been associated with a decrease in adverse events and toxicity symptoms, although neuropsychological toxicity may persist longer due to long term therapy [8, 34-37]. In patients (n=108) initiating an EFV-based regimen, stepped-dose versus full dose EFV were proven similarly efficient in a randomised trial [38]. In the present study, patients were not selected for adverse events; we observed nonetheless a significant reduction in anxiety scores and a trend in lower stress scores in patients on reduced dosing. No change in sleep quality or length as measured by a standardized questionnaire was identified. However, on study completion, all patients without exception chose to remain on the reduced dose, and this despite the intake of one additional pill for those receiving 400 mg QD (two 200 mg pills), compared to the standard treatment (one 600 mg pill).When considering the lifelong treatment, the reduction in drug exposure might translate into a decreased number of side effects.

The impact on overall drug costs can also be relevant; 600 mg pills cost 499 USD/month in Switzerland, whereas the cost of the 400 mg daily dose is 299 USD/month, and of

200 mg QD 166 USD/month. We have assumed two TDM assessments (278 USD) and a comprehensive genetic analysis (200 USD). The economic analysis reveals a cost saving of the 400 mg-dose reduction of 1857 USD/per year per patient, and of 3453 USD/per year per patient for a 200 mg-dose reduction; within the Swiss HIV Cohort Study (SHCS), which comprises more than 80% of all HIV-infected patients followed-up in Switzerland, if 15% of the patients on EFV-based regimen had a dose reduction down to 400 mg, and 10% to 200 mg according to TDM, the overall saving could be as high as 1'178'101 USD per year. EFV is not the only drug where cost could be decreased by lowering drug dosage; studies are ongoing with lamivudine, lopinavir/r and zidovudine for example [39].

Drug dosage reduction could have been predicted with nearly similar precision using genetic testing, instead of therapeutic drug monitoring, although the post-hoc nature of the *CYP2B6* genetic characterization and the fact that no full genetic characterization was provided for EFV-tolerant patients not included in the study, might soften this statement. The relative benefit of using one or the other strategy is unclear, considering that predictive dose reduction based on patient genetic profile differed from dose reduction according TDM in our study for 3 patients only. One patient underwent an EFV dose reduction to 200 mg/day lower than predicted by the genotyping results (400 mg/day), and reached the lowest EFV concentration on week 24 in the study. Two patients underwent an EFV dose reduction to 400 mg/day despite genotyping prediction (to 200 mg/day), and reached the highest EFV plasma levels on week 24. Thus, it is conceivable that all three patients would have reached the recommended target therapeutic range through genotyping. Still, further studies are needed to confirm the predictive potential of genetic testing, and, more specifically, to compare strategies to better identify the patients most likely to benefit from drug dose reduction.

We recognize several limitations to our study; firstly the limited sample size does not allow generalization of our findings to different population groups. Secondly, there was not randomization, and all patients benefited from a drug dose reduction. Thirdly, per protocol, patients had maximum two cycles of dose reduction to reach plasma concentration within target (1000-4000 ng/mL). In our experience, all patients but one fell into the drug plasma concentration target after the first cycle of dose reduction. For patients who reached the upper part of the therapeutic target range (i.e. 3000-4000 ng/mL), there is no evidence of a clinical benefit to expect from a second dose reduction.

The observation of larger than proportional reductions in concentration after dose reduction might indicate some non-linearity in EFV clearance, as well as non-specific regression to the mean, justifying our Bayesian approach to EFV TDM, and further non-captured influences (e.g. diet or interacting comedications).

In conclusion, TDM guided dose reduction in this an in other studies [12, 13] appears safe and should be considered in patients with high EFV concentrations; EFV dose reduction also prevents toxicities and minimizes treatment cost. This is also of particular interest for resource limited settings as WHO recently issued revised recommendations suggesting that EFV be the preferred regimen.

5.1.6. References

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5.2. *In vivo* analysis of efavirenz metabolism in individuals with impaired *CYP2A6* function

As we have seen, therapeutic drug monitoring of efavirenz (EFV) is supported by the high inter-individual and low intra-individual variability in plasma concentrations, as well as the potential relationships between treatment failure and side effects with plasma levels.
Besides gender and ethnicity, genetic variations in *CYP2B6* explain to a large extent the inter-individual variability in EFV exposure. But there is still a remaining variability, particularly in patients with impaired CYP2B6 function, suggesting a participation of other genetic and environmental influences.

EFV is extensively metabolised into hydroxylated metabolites with subsequent glucuronidation of these metabolites. Up to date, 8 metabolites have been reported, of which 3 are primary metabolites (8-hydroxy-EFV, 7-hydroxy-EFV and N-glucuronide-EFV) and 6 are secondary metabolites. 8-hydroxy-EFV is the main metabolite resulting of CYP2B6 activity, whereas 7-hydroxy-EFV is the second most important metabolite, produced mainly by *CYP2A6*, less by *CYP2B6*.
We have thus hypothesized that in patients with impaired CYP2B6 function, functional polymorphism in *CYP2A6* could play an important role.

The present study allowed a full reassessment of the proposed metabolic pathways, including the demonstration of the dependence on accessory pathways and their marginal capacity to respond to the absence of CYP2B6.

This is a collaborative research project realised jointly by J. di Iulio, M. Arab-Alameddine and A. Fayet as part of their respective PhD thesis.
Own contribution: A. Fayet developed the analytical method for drug and metabolites measurements, carried out all analyses in patients plasma samples, and participated to the interpretation of the data and the drafting of the article.

Related Appendices: 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9

IN VIVO ANALYSIS OF EFAVIRENZ METABOLISM IN INDIVIDUALS WITH IMPAIRED *CYP2A6* FUNCTION

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pdf version: Appendix 5.10.

5.2.1. Abstract

The antiretroviral drug efavirenz (EFV) is extensively metabolized into three primary metabolites: 8-hydroxy-EFV (8-OH-EFV), 7-hydroxy-EFV (7-OH-EFV) and N-glucuronide-EFV (N-gln-EFV). There is a wide inter-individual variability in EFV plasma exposure, explained to a great extent by CYP2B6, the main isoenzyme responsible of EFV metabolism and involved in the major metabolic pathway (8-hydroxylation) and to a lesser extent in 7-hydroxylation. When CYP2B6 function is impaired, the relevance of CYP2A6, the main isoenzyme responsible of 7-hydroxylation may increase. We hypothesize that genetic variability in this gene may contribute to the particularly high, unexplained variability in EFV exposure in subjects with limited CYP2B6 function. This study characterized *CYP2A6* variation (14 alleles) in individuals (n=169), previously characterized for functional variants in *CYP2B6* (18 alleles). Plasma concentrations of EFV and its primary metabolites (8-OH-, 7-OH-, and N-gln-EFV) were measured in different genetic backgrounds *in vivo*. Results indicate a critical role of the accessory metabolic pathway CYP2A6 in limiting drug accumulation in CYP2B6 slow metabolizers. Dual CYP2B6 and CYP2A6 slow metabolism will occur at significant frequency in various human populations, leading to extremely high efavirenz exposure.

5.2.2. Introduction

The antiretroviral drug efavirenz (EFV) is primarily metabolized by the cytochrome P450 2B6 (CYP2B6) [1]. There is high inter-individual variability in plasma drug levels after drug administration at the usual dosage regimen of 600 mg/day [2]. This observation reflects the existence of slow and rapid metabolizer phenotypes associated with genetic variations in *CYP2B6* [3-7]. Although *CYP2B6* alleles explain to a large extent the observed inter-individual variability in EFV exposure, there is a remaining variability, in particular among individuals with impaired CYP2B6 function [5]. This unexplained variability suggests the participation of other genetic and environmental influences in EFV elimination.

EFV is extensively metabolized into three primary metabolites; two of them are hydroxylated metabolites [8-hydroxy-EFV (8-OH-EFV) and 7-hydroxy-EFV (7-OH-EFV)] and the third is a glucuroconjugated product [N-glucuronide-EFV (N-gln-EFV)] (Figure 1). 8-hydroxylation is the main metabolic pathway (92%) and is essentially the result of CYP2B6 activity [1]. Recent *in vitro* data suggests that 7-hydroxylation is the second most important pathway (<8%) and is mainly due to CYP2A6 activity and to a lesser extent to CYP2B6 activity [8] (Figure 1). N-gln-EFV has been identified in human plasma

and urine although the pathway has not been characterized [9] (Figure 1). Here we hypothesize that, in individuals with impaired CYP2B6 function, functional polymorphism in *CYP2A6* may be of clinical importance.

CYP2A6 has been shown to have large inter-individual and interethnic variability in levels of expression and activity, and this is thought to be largely due to genetic polymorphisms [10]. Variation in activity affects the metabolism of CYP2A6 substrates such as nicotine, and coumarin.

In this study, we conducted detailed assessment of functional genetic variation in *CYP2A6* in a well characterized population of HIV-infected individuals receiving efavirenz, and validated the observed genotypic/phenotypic associations by *in vivo* metabolite profiling.

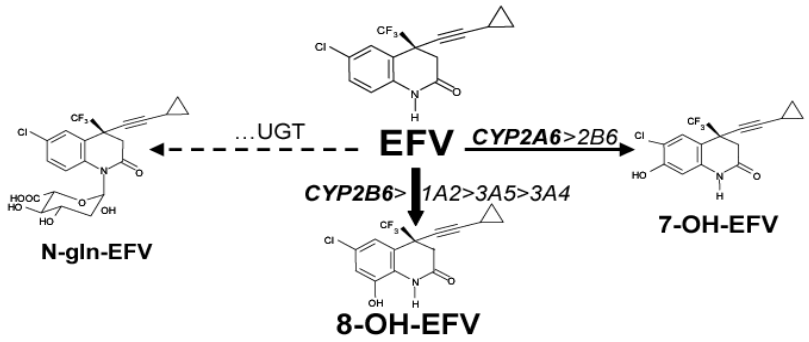


Figure 1: EFV primary metabolic pathways.
EFV is extensively metabolized into three primary metabolites: 8-hydroxy-EFV (8-OH-EFV), 7-hydroxy-EFV (7-OH-EFV) and N-glucuronide-EFV (N-gln-EFV)

5.2.3. Materials and methods

5.2.3.1. Study population

The study group includes 169 individuals extensively characterized for their *CYP2B6* genotype in a previous study [5], and described in Supplementary Table S1 (Appendix 5.3.). The ethics committees of all participating center approved the genetics project and participants gave written informed consent for genetic testing.

5.2.3.2. Population pharmacokinetics

A population pharmacokinetic analysis of EFV was fitted according to our previous study [11] to derive average population parameters and inter-patient variability and to estimate the influence of demographic factors on its elimination. EFV plasma levels were

collected as part of therapeutic drug monitoring protocol according to local treatment guidelines. EFV pharmacokinetics was characterized using a one-compartment model with first order absorption from the gastrointestinal tract and assigned an inter-individual variability on oral clearance (CL), assuming a log-normal distribution with mean zero and variance Ω. The influence of demographic covariates (gender, ethnicity, age, body weight, height and other co-medications such as antiretroviral drugs and few other medications) was analyzed using linear relationships. A proportional error model with a mean of zero and a variance of σ^2 was used to describe intra-individual variability. Individual Bayesian estimates of CL were used to derive individual area under the curve (AUC=Dose/CL) that were used for statistical analyses. The population pharmacokinetics analysis was performed with NONMEM® (version VI, NM-TRAN version II) using FOCE INTERACTION to fit the models (Ref NONMEM). Model choice was based on the likelihood ratio test, and goodness of fit plots.

5.2.3.3. *CYP2A6* genotyping

Analysis of *CYP2A6* genetic variation included extensive genotyping of known alleles, full re-sequencing for selected individuals for the identification of new alleles, and study of copy number variation. Genotyping targeted all alleles with known impact on expression or function in Caucasians, as well as the most common decrease/loss-of-function (DOF/LOF) alleles found in Africans and Asians (Table 1, and references therein). We used TaqMan allelic discrimination (ABI prism 7000 sds software, Applied Biosystems) to investigate 5 alleles (**2, *9, *13, *15, *17*). Alleles resulting from gene conversion (**5, *7, *10, *12, *19*) and promoter alleles (**1H,*1J*) were genotyped by sequencing. Primers and probes are shown in Supplementary Table S2 (Appendix 5.4.).

On account of the working hypothesis that *CYP2A6* variation would be clinically relevant among individuals with impaired CYP2B6 metabolism, *CYP2A6* promoter region, and all 9 exons and intron-exon boundaries (3775 bp) were fully re-sequenced in all individuals homozygous for a LOF of *CYP2B6* (n=23). Primers are shown in Supplementary Table S3 (Appendix 5.5.).

Gene copy number was determined in all individuals, as gene deletions (**4A-4F*) and gene duplications (**1X2A-B*) have been described for this gene [13, 22-25]. Real-time PCR was performed using the TaqMan ABI prism 7000 sds software (Applied Biosystems). For each analysis, standard curves (seven serial 1:2 dilutions of genomic DNA) were built for a reference gene (β-*Globin*, two copies per diploid genome), and for *CYP2A6* in separate wells. β-*Globin* primers and probe were previously published [26]

(Supplementary Table S2, Appendix 5.4.). *CYP2A6* primers and probes were obtained as Assay-on-Demand from Applied Biosystems (TaqMan® Gene Expression Assays: HS0001002_cn) (Supplementary Table S2, Appendix 5.4.). The probe is located in exon 7, which is missing in the gene deletions described in the literature [22-24].

Table 1: *CYP2A6* Alleles investigated

Allele	Functional SNP (rs)	Proposed functional consequence	Ref
2A6*1H,*1J	g.-745A>G (rs N.A.)	DOF (<50% decreased activity *in vitro*).	[12]
2A6*1X2A-B	Gene duplication	GOF (increased activity *in vivo*).	[13]
2A6*2	g.1799T>A (rs 56844942)	LOF (catalytically inactive).	[14]
*2A6*4A-F*	GC with *CYP2A7* in intron 8	LOF (gene deletion).	[15, 16]
*2A6*5*	g.6582G>T (rs 5031017)	LOF (catalytically inactive).	[17]
2A6*7,*10,*19	g.6558T>C (rs 5031016)	DOF (≥50% decreased activity *in vitro*).	[18]
*2A6*9,*13,*15*	g.-48T>G (rs 28399433)	DOF (≥50% decreased activity *in vivo/vitro*).	[19]
2A6*12	GC with *CYP2A7* in intron 2	DOF (≥50% decreased activity *in vivo/vitro*).	[20]
*2A6*17*	g.5065G>A (rs 28399454)	DOF (≥50% decreased activity *in vivo/vitro*).	[21]
*2A6*34*	GC with *CYP2A7* in intron 4	LOF (partial gene deletion)	This study

SNP, Single Nucleotide Polymorphism. DOF, decrease-of-function allele. LOF, loss-of-function allele. GOF, gain-of-function allele. GC, gene conversion.

5.2.3.4. *CYP2B6* genotyping

The details of *CYP2B6* genotyping have been presented previously [5].

5.2.3.5. Nomenclature

CYP2A6 allele designation was performed on the basis of genotyping of known functional single nucleotide polymorphisms (SNPs), as well as by full re-sequencing. Novel alleles were designated in concordance with the CYP Allele Nomenclature Committee (http://www.cypalleles.ki.se). Position numbering refers to genomic DNA (indicated as g.) according to NT_011109.15 (bp1=A of ATG of *CYP2A6*). *CYP2B6* allele designation was established as before [5]. *CYP2A6* investigated alleles were reported as decrease-of-function alleles (DOF) or loss-of-function alleles (LOF), according to the

known/proposed activity of the encoded protein. The DOF alleles were further broken down into two categories: DOF<50% (less than 50% decrease in activity or expression) and DOF≥50% (more or equal to 50% decrease in activity or expression) (Table 1).

5.2.3.6. *In vivo* metabolites analyses

Plasma concentrations of EFV, 8-OH-EFV, 7-OH-EFV and N-gln-EFV were determined by liquid chromatography coupled to triple quadripole tandem mass spectrometry (LC-MS/MS, Thermo Scientific Corporation San Jose, USA) after protein precipitation with acetonitrile using an adaptation of our previously reported method [27] (See Appendix 5.9). 8-OH-EFV-d_4 and 8-OH-EFV were obtained from Toronto Research Chemicals Inc. (North York, Canada). The *m/z* transition, collision energy (V) and tube lens used for 7-OH-EFV and 8-OH-EFV in the Selected Reaction Monitoring (SRM) Mode were 330.0 → 257.9, 31 and 97, respectively. N-gln-EFV was analysed in the SIM mode (m/z = 490.0). Chromatographic profiles and LC-MS spectra of EFV metabolites were compared with available data [1, 9]. Quantitative analysis of EFV and 8-OH-EFV were performed with matrix-matched calibration samples using the internal standard method (EFV-d_4 and 8-OH-EFV-d_4, respectively), while 7-OH-EFV and N-gln-EFV levels were expressed in arbitrary units (a.u.) using signal peak areas. The lower limit of quantification is 50 ng/ml for EFV and 8-OH-EFV. Values were adjusted by dose intake for three individuals that were not receiving a 600 mg EFV regimen.

5.2.4. Results

5.2.4.1. Population pharmacokinetics

393 EFV plasma concentrations provided by 169 HIV infected individuals (range 0.1 – 59.4 μg/ml) were analysed. Average CL was 11.3 L/h with an inter-individual variability of 65 % (CV %), volume of distribution (V) was 388 L and the absorption constant (ka) 0.62 h^{-1}. Body weight was retained as significant demographic factor, yielding a 70% increase in CL on body weight doubling; it accounted for the effect of height, age and gender and explained 3% of CL variability. Individual AUC values ranged between 15.4 and 436.0 (μg*h/ml). Drug interactions were ruled out as significant contributor to EFV pharmacokinetics in this study, as the presence of ritonavir revealed no statistically significant effect on EFV exposure and only few individuals were co-administered a non antiretroviral CYP inducer (n=2) or inhibitor (n=4) drug.

Table 2: Frequency of *CYP2A6* genotypes in different ethnicities

Region	Allele(s)	SNP	All subjects [%] (n)	Caucasians [%] (n)	Africans [%] (n)	Hispanics [%] (n)	Asians [%] (n)
5' UTR	**1H, *1J*	-745A>G					
		AA	79.8 (134)	81.6 (115)	62.5 (10)	100.0 (7)	50.0 (2)
		AG	20.2 (34)	18.4 (26)	37.5 (6)	0.0 (0)	50.0 (2)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
5' UTR	**9, *13, *15*	-48T>G					
		TT	83.9 (141)	81.6 (121)	62.5 (10)	85.7 (6)	100.0 (4)
		TG	15.5 (26)	18.4 (20)	31.3 (5)	14.3 (1)	0.0 (0)
		GG	0.6 (1)	0.0 (0)	6.2 (1)	0.0 (0)	0.0 (0)
Exon 3	**2*	1799T>A					
		TT	96.4 (162)	96.5 (136)	100.0 (16)	85.7 (6)	100.0 (4)
		TA	3.6 (6)	3.5 (5)	0.0 (0)	14.3 (1)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 7	**17*	5065G>A					
		GG	98.2 (166)	100.0 (141)	81.3 (13)	100.0 (7)	100.0 (5)
		GA	1.8 (3)	0.0 (0)	18.7 (3)	0.0 (0)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	**7, *10, *19*	6558T>C					
		TT	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		TC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		CC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	**5*	6582A>G					
		AA	100.0 (169)	100 (141)	100.0 (16)	100.0 (7)	100.0 (5)
		AG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 2	**12*	GC with *CYP2A7*					
		No GC	98.2 (165)	98.6 (139)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.8 (3)	1.4 (2)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 4	**34*	GC with *CYP2A7*					
		No GC	99.4 (168)	100.0 (141)	100.0 (16)	100.0 (7)	80.0 (4)
		Het GC	0.6 (1)	0.0 (0)	0.0 (0)	0.0 (0)	20.0 (1)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8 –> 3'UTR	**4A-F* (deletion)	GC with *CYP2A7*					
		No GC	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		Het GC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8 –> 3'UTR	*1X2A-B (duplication)	GC with CYP2A7					
		No GC	98.8 (167)	99.3 (140)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.2 (2)	0.7 (1)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)

SNP, single nucleotide polymorphism. UTR, untranslated region. GC, gene conversion. Het, heterozygous. Hom, homozygous. The individual genotype frequency was calculated based on the number of individuals for whom genotyping for the selected SNPs has been successfully performed (n). SNP position is based on genomic (g.) DNA numbering (bp1=A of ATG).

5.2.4.2. *CYP2A6* genotyping

Fifty-five (33%) individuals carried one *CYP2A6* DOF or LOF allele, and 13 (8%) carried two DOF and/or LOF alleles (Table 2). Analysis of copy number identified three individuals heterozygous for a gene deletion and two individuals heterozygous for gene duplication (Figure 2A). These two individuals with a *CYP2A6* gene duplication (expected to represent a gain-of-function allele) were included in the group of extensive CYP2A6 metabolizers.

CYP2A6 was fully re-sequenced in the 23 individuals homozygous for a LOF allele of *CYP2B6* (slow metabolizers). *CYP2A6* re-sequencing identified one polymorphism in the promoter region, 16 polymorphisms in exons and 25 polymorphisms in exon-intron boundaries. The promoter SNP g.-48T>G (*CYP2A6*9, *13, *15*), modifies the TATA box leading to a decreased expression of the gene [19, 28, 29]; five exonic SNPs are non-synonymous, two of which are associated with a decrease-of-function (g.5065G>A [V365M], in *CYP2A6*17* [21]; g.6558T>C [I471T], in *CYP2A6*7, *10, *19* [18]) (Figure 2B). In addition, re-sequencing identified an individual that carried a deletion of the first four exons of *CYP2A6*. This new partial gene deletion could not have been identified by gene copy number determination using a probe in exon 7. The new allele results from an unequal crossover in intron 4 between *CYP2A7* and *CYP2A6* (Figure 3A, B). Primers used for identification of the new allele are shown in Supplementary Table S4 (Appendix 5.6.). Further analyses concluded that the patient carried both a known deletion (allele **4A*) on one chromosome, as well as the new allele (*CYP2A6*34,* GenBank accession: EU814898) on the second chromosome. The new allele has ten amino acid substitutions characterizing the DOF allele **12* [20] and substitutions R128L and S131A present in the LOF allele **26* [24], as well as additional non-synonymous variants. Thus, this partial gene deletion was considered a LOF allele.

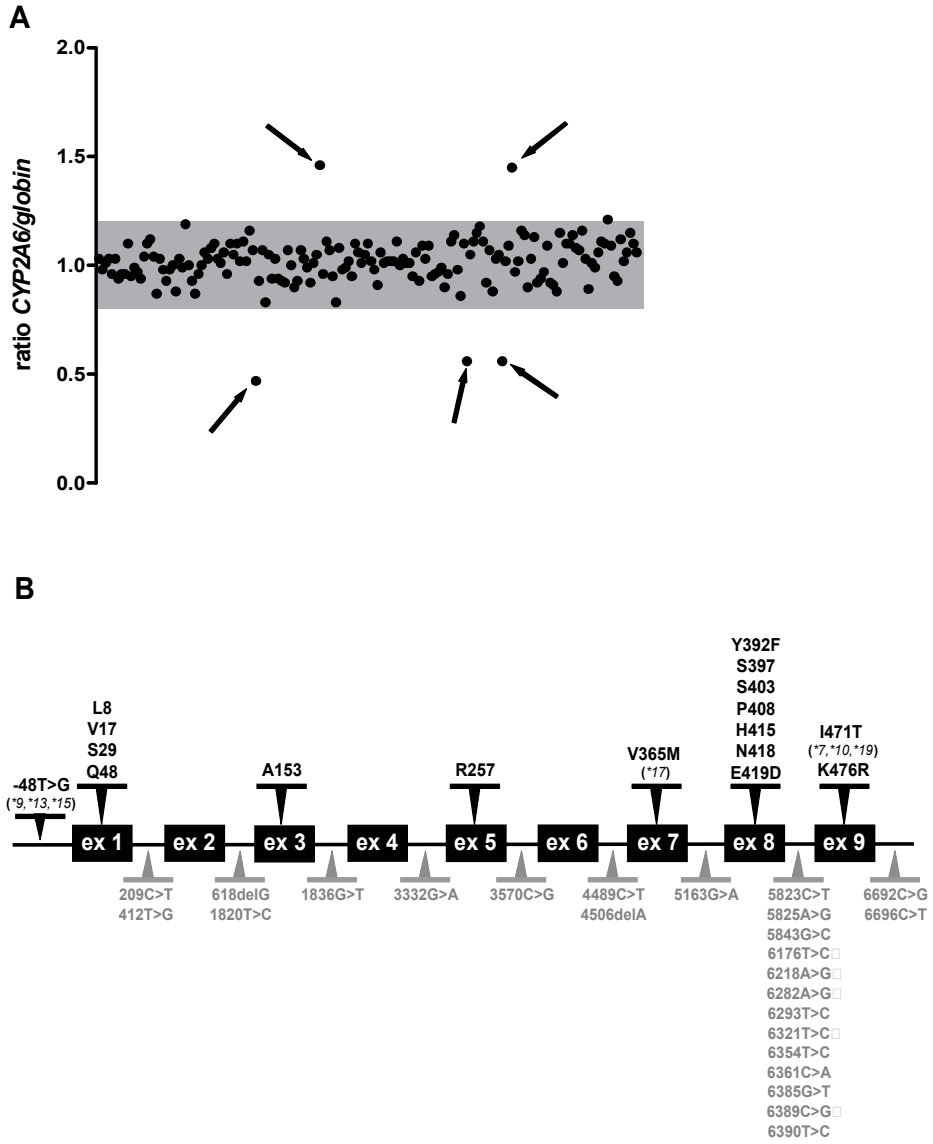


Figure 2: *CYP2A6* genotyping.

Panel A. Gene copy number determination. Each dot represents one individual (n=169). The grey box represents the CYP2A6/β-Globin ratio interval for individuals with two *CYP2A6* copies (ratio average value=1.02, percentile 95%, 0.87 to 1.16). Black arrows indicate the individuals heterozygous for a gene deletion (*CYP2A6*4A-F*) (ratio 0.47/0.56/0.56 respectively from left to right) and individuals heterozygous for a gene duplication (*CYP2A6*1X2A*-B) (ratio 1.47/1.45 respectively from left to right). Panel B. SNPs identified by re-sequencing *CYP2A6* in the individuals *CYP2B6* hom LOF (n=23). There is one DOF SNP (-48T>G) in the promoter region (TATA box), eleven synonymous SNPs, three non-synonymous SNPs and two non-synonymous DOF SNPs (V365M, I471T) in the exons, and 25 SNPs in the intron-exon boundaries. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. Ex., exon. DOF, decrease-of-function. LOF, loss-of-function. SNP, Single Nucleotide Polymorphism. Hom, homozygous.

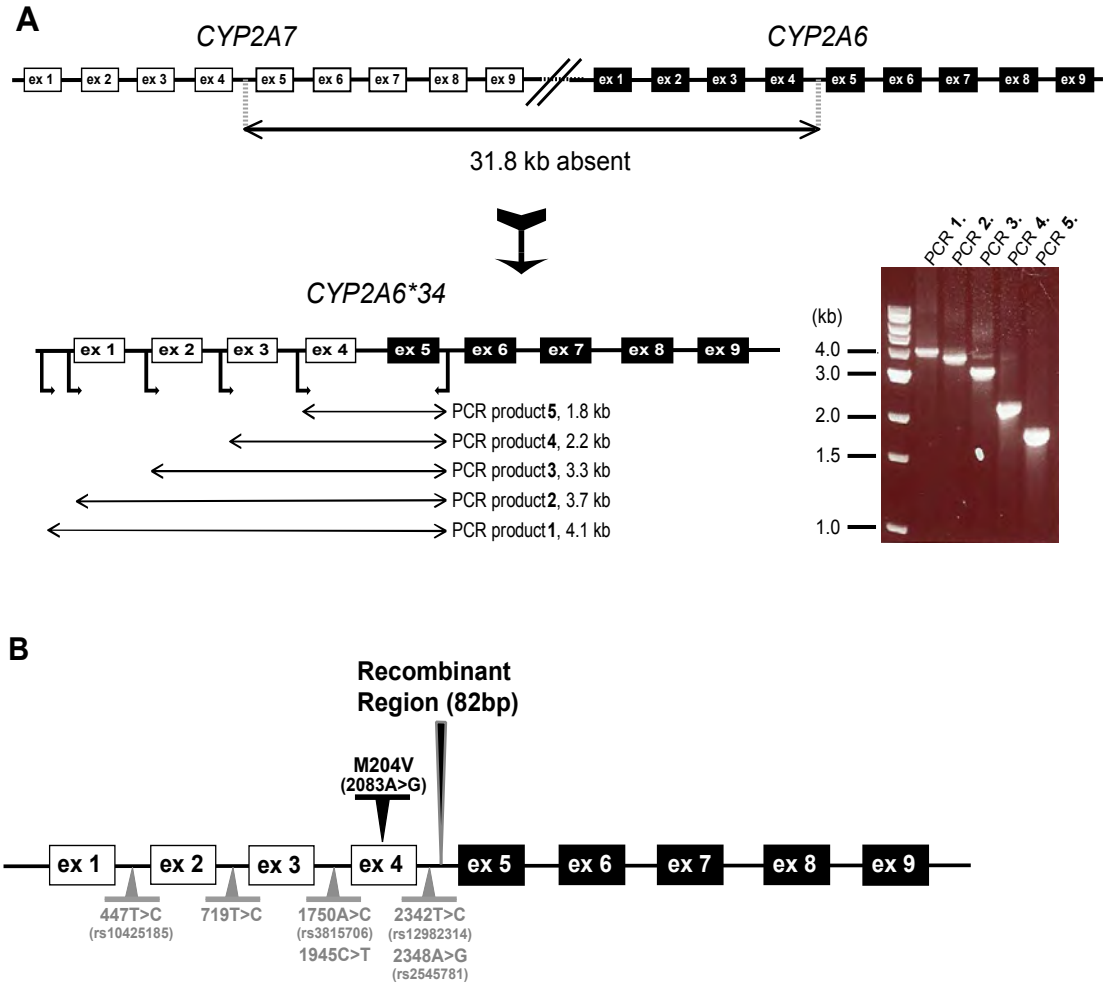


Figure 3: *CYP2A6*34* identification and characterization.
Panel A. Identification of a new hybrid allele (*CYP2A6*34*). PCRs were run with forward primers specific to *CYP2A7* and reverse primer specific to *CYP2A6*. Panel B. SNPs identified by re-sequencing all exons and intron-exon boundaries of *CYP2A6*34*. White boxes represent exons of *CYP2A7* origin; Black boxes represents exons of *CYP2A6* origin. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. Ex., exon. DOF, decrease-of-function. LOF, loss-of-function. SNP, Single Nucleotide Polymorphism. Hom, homozygous.

5.2.4.3. Association of CYP2A6 genotype and EFV plasma exposure

Without consideration for CYP2B6 function, *CYP2A6* alleles function appear to have a limited effect on EFV exposure (Figure 4A). Individuals with reference alleles had a median log10 EFV AUC of 1.67 μg*h/ml, individuals carrying one DOF or LOF allele had a median log10 EFV AUC ranging from 1.64 to 1.68 μg*h/ml (p=0.29 versus reference alleles), whereas individuals carrying two DOF and/or LOF alleles had a median log10 EFV AUC ranging from 1.75 to 2.99 μg*h/ml (p=0.002 versus reference alleles).

Because of the working hypothesis that *CYP2A6* genotype would be clinically relevant among individuals with impaired CYP2B6 metabolism, data were stratified according to the number of *CYP2B6* functional alleles. As this stratification reduced the number of individuals per category, all *CYP2A6* DOF and LOF alleles were referred here as D/LOF alleles in order to increase the power (the raw results are shown in Supplementary Table S5, Appendix 5.7.). The contribution of *CYP2A6* alleles was particularly relevant among individuals CYP2B6 slow metabolizers. Here, individuals with common *CYP2A6* alleles presented lower median log10 EFV AUC values of 2.18 μg*h/ml, compared to individuals carrying one D/LOF allele (2.28 μg*h/ml), and individuals homozygous for a D/LOF allele (2.48 μg*h/ml), p=0.06 (Figure 4B).

5.2.4.4. *In vivo* metabolites analyses

To support the genotyping results, we assessed the concentration of 8-OH, 7-OH and N-gln-EFV in different genetic backgrounds *in vivo*. For this analysis, we identified individuals (n=48) representative of the various genetic profiles (Supplementary Table S6, Appendix 5.8.). Chromatogram profiles of individuals with 3 different genetic backgrounds are shown in Figure 5A, B, C.

CYP2B6 slow metabolizers, when compared to CYP2B6 extensive metabolizers, presented a 11% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 6% decrease in median log10 7-OH-EFV (a.u.) – consistent with a role of CYP2B6 in this pathway (Figure 1) – and a 35% increase in the median log10 N-gln-EFV (a.u.). The limited absolute decrease in 8-OH-EFV and 7-OH-EFV concentration was the result of the significant increase of the parent compound. When results were expressed as ratios, we observed a 67% decrease in median log10 8-OH-EFV/EFV ratio, a 33% decrease in median log10 7-OH-EFV/EFV ratio, and a 5% decrease in the N-gln-EFV/EFV ratio.

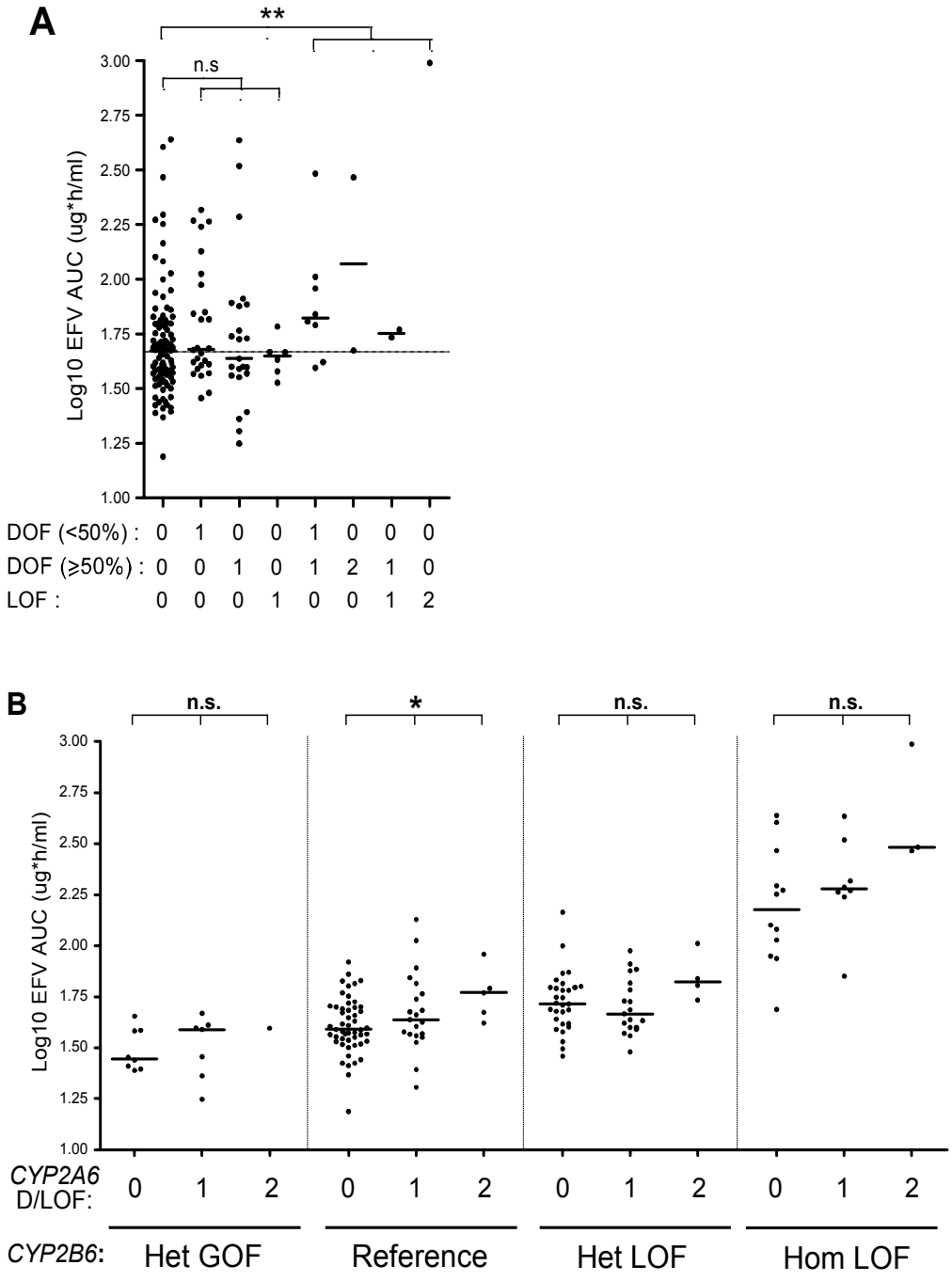
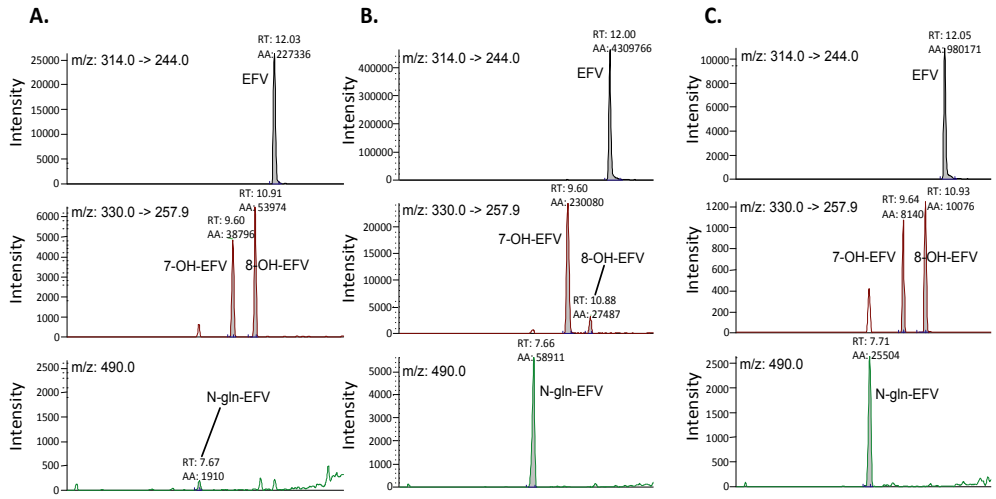


Figure 4: Association of *CYP2A6* genotype and EFV plasma exposure.
Panel A. Individuals were classified according to their number of *CYP2A6* DOF<50%, DOF≥50% and/or LOF alleles. Panel B. Data were stratified according to *CYP2B6* genotype and according to the number (0, 1 or 2) of *CYP2A6* DOF and/or LOF alleles, all referred here as D/LOF due to the small number of individuals in each category. GOF, gain-of-function. Reference, reference alleles. DOF, decrease-of-function. LOF, loss-of-function. Het, heterozygous. Hom, homozygous. *, $p<0.05$, **, $p<0.005$. n.s., non significant.



CYP2B6:	Reference	Hom LOF	Hom LOF
CYP2A6:	Reference	Reference	Hom DOF

Figure 5: Efavirenz metabolite profiling.
Plasma concentrations of EFV, 7-OH-EFV, 8-OH-EFV and N-gln-EFV were determined by liquid chromatography coupled with triple quadripole tandem mass spectrometry. Shown are chromatographic profiles of plasma samples from three individuals with representative genotypes. Panel A. reference alleles for *CYP2B6* and *2A6*. Panel B. hom LOF *CYP2B6* (**6/*6*), and reference alleles for *CYP2A6*. Panel C. hom LOF *CYP2B6* (**6/*18*) and hom DOF *CYP2A6* (**9/*9*). Note that the scale varies for the different profiles. D/LOF, decrease/loss-of-function. Hom, homozygous. Reference, reference alleles.

Carriers of one and two *CYP2A6* D/LOF alleles, when compared to CYP2A6 extensive metabolizers, were associated respectively with a 1% and 6% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 1% increase and a 2% decrease in median log10 7-OH-EFV (a.u.) and a 0% and 3% decrease in the median log10 N-gln-EFV (a.u.).

Data were then stratified according to the number of *CYP2B6* functional alleles. The contribution of *CYP2A6* alleles was more relevant among *CYP2B6* slow metabolizers. In this group, carriers of one and two *CYP2A6* D/LOF alleles, when compared to *CYP2A6* extensive metabolizers, presented, respectively, a 8% decrease and a 3% increase in the median log10 8-OH-EFV concentration (ng/ml), a 2% and 22% decrease in median log10 7-OH-EFV (a.u.) and a 6% and 20% increase in the median log10 N-gln-EFV (a.u.) (Figure 6A, B, C). These results are consistent with the role of CYP2A6 as an alternative pathway in EFV metabolism, and the role of N-glucuronidation in the setting of multiple D/LOF in main and accessory hydroxylating pathways.

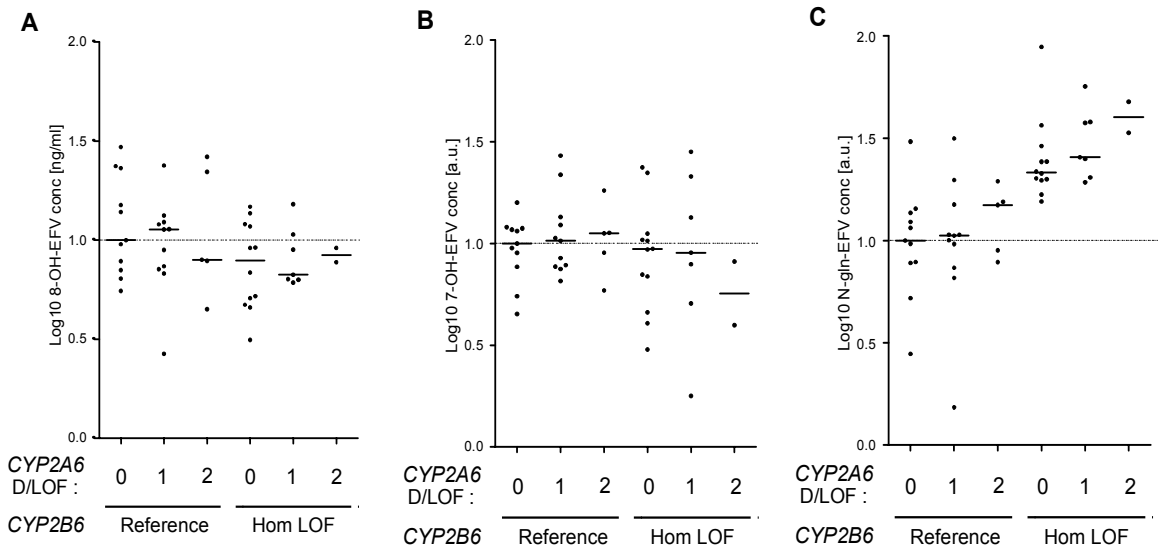


Figure 6: EFV metabolite concentration in individuals with different *CYP2B6* and *CYP2A6* genetic profiles.
EFV metabolite concentrations were stratified according to *CYP2B6* genotype (Reference or Hom LOF) and according to the number (0, 1 or 2) of *CYP2A6* D/LOF alleles. Panel A. 8-OH-EFV concentration [ng/ml]. Panel B. 7-OH-EFV concentration [a.u.]. Panel C. N-gln-EFV concentration [a.u.]. The median log10 value for CYP2B6 and CYP2A6 extensive metabolizers is normalized to 1. Reference, reference alleles. D/LOF, decrease/loss-of-function. Hom, homozygous.

5.2.5. Discussion

Detailed genetic analysis of EFV metabolic pathways allows a better understanding of the inter-individual variability in EFV plasma exposure. It highlights the critical role of the accessory metabolic pathways in limiting drug accumulation in individuals characterized as CYP2B6 slow metabolizers.

We performed a comprehensive assessment of *CYP2A6* genetic variations because of the a role of this isoenzyme in the *in vitro* formation of 7-OH-EFV [8]. Analysis included a large number of functional alleles associated with impaired CYP2A6 function, and the assessment of gene copy number. In addition, we identified a new functional genetic variant in this locus through gene re-sequencing that results from the recombination of *CYP2A7* and *CYP2A6.* Overall 70 of 169 study participants carried one or more D/LOF alleles or copy number variants. The pharmacokinetic data confirmed to a large extent the reported or predicted functional effect of these variants.

Analysis of the primary EFV metabolites *in vivo* showed results consistent with the predicted balance among the main and accessory hydroxylating pathways. LOF *CYP2B6* alleles were associated with a decrease in EFV hydroxy metabolites as previously reported by *in vitro* studies [8], whereas increased levels of N-gln-EFV were observed indicating the redirection of the metabolism through this accessory pathway. Among CYP2B6 extensive metabolizers, *CYP2A6* D/LOF alleles did not modify EFV 7-hydroxylation because this pathway is still supported by CYP2B6 in the absence of CYP2A6 activity [8]. However, in the presence of impaired CYP2B6 function, *CYP2A6* D/LOF alleles were associated with a further decrease in 7-OH-EFV - consistent with the hypothesis that this alternative pathway becomes increasingly relevant in the context of impaired *CYP2B6* function - and with a marked increase in N-gln-EFV. Analysis of N-gln-EFV is particularly reliable because it is not further metabolized in humans, and the concentration is not dependent on other isoenzyme genetic profiles [9].

This study is limited by the incomplete understanding of functional variations in other genes involved in EFV metabolism. We could not investigate variations of the gene(s) involved in N-glucuronidation due to lack of information on the UGT isoenzyme responsible of this step, and the large number of members in the family. Although CYP1A2 was reported to play a role in EFV metabolism, leading to 8-OH-EFV [1], report on functional alleles has been mostly limited to Asians (http://www.cypalleles.ki.se; [30]). There is a paucity of functional alleles in *CYP3A4* despite extensive investigation by many groups over the years [31], and the low frequency of functional polymorphisms found in the coding regions can not account for the variation observed [32]. In addition, a number of factors may affect CYP3A expression, tissue-specific splicing, variable control of gene transcription by endogenous and exogenous molecules, and genetic variations in proteins that regulate CYP3A expression through nuclear hormone receptors [33].

The present work indicates that the presence of multiple LOF alleles at both the main (CYP2B6) and accessory (CYP2A6) hydroxylation pathways results in extremely high EFV exposure. The clinical relevance of dual CYP2B6 and CYP2A6 slow metabolism is determined by the frequency of decrease/loss-of-function alleles in various human populations. Indeed, the loss-of-function allele *CYP2B6*6* can reach very high frequencies in the population (up to 26% in Caucasians, 47% in Africans, and 18% in Asians) [5, 34]. Similarly, the prevalence of *CYP2A6* alleles lacking or showing reduced enzymatic activity is elevated in the various populations (9% in Caucasians, 22% in

Africans and up to 50% in Asians) ([35] and this study). Therefore, dual CYP2B6 and CYP2A6 slow metabolism will occur at significant frequency in various populations, leading to extremely high EFV exposure in a relevant proportion of individuals. A formal population pharmacokinetic-pharmacogenetic analysis of the data is presented in a separate publication [36].

The approach of identifying functional alleles in multiple metabolic pathways, in combination with metabolite assessment *in vivo* can be of general interest for the validation of *in vitro* studies for other drugs, and to complement pharmacological analysis during drug development.

5.2.6. References

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5.3.Pharmacogenetics-based population pharmacokinetic analysis of efavirenz in HIV-1-infected individuals

Population-based approaches represent a highly suitable way to capture the contribution of multiple influences to a phenotype and to quantify both between and within patients variances, which constitutes one of the main rationales for studies on the identification of genetics determinants and adequate characterisation of pharmacokinetic (PK) or pharmacodynamic phenotypes.

The comprehensive understanding of the genetic determinants of efavirenz PK model incorporating genetic covariants performed by Rotger *et al*. [1] has allowed us to develop and complete a pharmacogenetic-based population pharmacokinetic analysis of EFV with NONMEM program in 169 HIV-infected patients, in order to characterise the joint impact of genetic polymorphisms in the main (CYP2B6) and accessory (CYP2A6, 3A4/5) metabolic pathways involved in EFV elimination.
This original approach will serve as a proof-of-concept for further investigations of multiple genotype-phenotype interactions involving other antiretroviral drugs.

This is a collaborative research project realised jointly by M. Arab-Alameddine, J. di Iulio and A. Fayet as part of their respective PhD thesis.
Own contribution: A. Fayet was in charge of all efavirenz plasma levels measurements, collection of clinical information into the TDM database, and constitution of the dataset for population pharmacokinetic analyses.

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PHARMACOGENETICS-BASED POPULATION PHARMACOKINETIC ANALYSIS OF EFAVIRENZ IN HIV-1-INFECTED INDIVIDUALS

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5.3.1. Abstract

Objective

Besides CYP2B6, other polymorphic enzymes contribute to efavirenz (EFV) interindividual variability. This study was aimed at quantifying the impact of multiple alleles on EFV disposition.

Method

Plasma samples from 169 human immunodeficiency virus (HIV) patients characterized for *CYP2B6, CYP2A6* and *CYP3A4/5* allelic diversity were used to build up a population pharmacokinetic model using NONMEM (Non-linear mixed effects modeling), the aim being to seek for a general approach combining genetic and demographic covariates.

Results

Average clearance (CL) was 11.3L/h with a 65% interindividual variability that was explained largely by *CYP2B6* genetic variation (31%). *CYP2A6* and *CYP3A4* had a prominent influence on CL mostly when *CYP2B6* was impaired. Pharmacogenetics fully accounted for ethnicity, leaving body weight as the only significant demographic factor influencing CL. Square roots of the numbers of functional alleles best described the influence each gene, without interaction.

Conclusion

Functional genetic variations in both principal and accessory metabolic pathways demonstrate a joint impact on EFV disposition. Therefore, dosage adjustment in accordance with the type of polymorphism (*CYP2B6*, *CYP2A6* and *CYP3A4*) is required in order to maintain EFV within the therapeutic target levels.

5.3.2. Introduction

Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, is widely used in combination with nucleoside inhibitors as first-line treatment of type I human immunodeficiency virus (HIV-1) infection. It is generally prescribed at a fixed dosage of 600 mg daily, despite the presence of a marked interindividual variability intendency to produce elevated plasma drug concentration levels (1-3) that have been shown to be associated with central nervous system toxicity (4-6).

EFV is metabolized primarily by *CYP2B6* and, to a lesser extent, by accessory pathways involving *CYP2A6*, *CYP3A4/3A5*, and uridine-glucuronyl-transferases (7-9). Several studies have shown that *CYP2B6* is highly polymorphic and that genetic variations play an important part in EFV plasma concentration variability (5;10-15). Genetic polymorphisms of the *CYP3A4|3A5* have also been associated with higher EFV exposure (6) but the influence of *CYP2A6* polymorphism on EFV pharmacokinetics has yet not been characterized. Considering the increasing number of allelic variants that are being described and the resulting complexity of allele combinations that could influence EFV elimination, we conducted a population pharmacokinetic analysis in HIV-1 infected individuals fully characterized for *CYP2B6*, *CYP2A6* and *CYP3A4/A5* genetic variation. Our main area of focus were (i) to assess the relative contribution of multiple functional alleles involved in EFV elimination along with other demographic or environmental factors, (ii) to characterize the nature of the relationship between individual allelic constitution and EFV disposition and (iii) to explore models for gene-gene interactions that could lead to a better understanding of the interrelationships of specific enzymes involved in EFV elimination.

5.3.3. Material and methods

5.3.3.1. Study population

A total of 169 HIV-1 infected individuals from the Swiss HIV Cohort Study were characterized for *CYP2B6, CYP2A6* and *CYP3A4/A5* genetic variation. EFV drug levels were measured during routine therapeutic drug monitoring according to local treatment guidelines. All participants gave their informed consent for genetic testing. A median of 1 concentration sample per individual (range 1-23) was collected and drawn between 0.6 and 38 hours after last drug intake under steady-state conditions.

5.3.3.2. Analytical method

Blood samples (5 ml) were collected into lithium heparin or EDTA-K Monovette syringes (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation, inactivated for virus at 60°C for 60 minutes, and stored at -20°C until analysis. Plasma EFV levels were determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) according to a validated method. The calibration curves are linear up to 10 µg/ml, with a lower limit of quantification of 0.1 µg/ml.

Table 1: Functional alleles evaluated in the study and genotype-based activity score classification

Functional alleles				
Functional consequence	CYP2B6 alleles	CYP2A6 alleles	CYP3A4 alleles	CYP3A5 alleles
Loss-of-function (LOF)	*11, *15, *28	*2, *4		*3, *6, *7,*10, *11
Diminish function (DOF)	*6, *18, *27, *29	*1H, *1J, *5, *7, *9, *10, *12, *13, *15, *17, *19, *34	rs4646437 *1B	-
>25%		*5, *7, *9, *10, *12, *13, *15, *17, *19, *34	-	-
<25%		*1H, *1J	-	-
Reference	*1, *2, *3, *5, *17	*1	*1	*1
Gain-of-function	*4, *22	*1X2	-	-

Genotypes and Activity Score Classification					
Score A	Alleles (allele1/alelle2)	Score B	Alleles (allele1/alelle2)	Score C*	Alleles (allele1/alelle2)
0	LOF/LOF LOF/DOF DOF/DOF	0	LOF/LOF	0	LOF/LOF
1	Ref /LOF Ref/DOF	0.25	LOF/DOF	0.25	LOF/DOF
2	Ref/Ref	0.5	Ref/LOF DOF/DOF	0.5	Ref/LOF
3	Ref/GOF	1	Ref/LOF	0.75	DOF/DOF
		1.5	Ref/DOF	1	Ref/DOF >25%
		2	Ref/Ref	1.5	Ref/DOF < 25%
				2	Ref/Ref

*Only for CYP2A6 alleles
Ref= reference allele, Hom= homozygous, Het= heterozygous, LOF= loss of function, DOF= diminished function, GOF= gain of function.

5.3.3.3. Nomenclature and Functional Score

Alleles are designated in concordance with the *CYP* Allele Nomenclature Committee. (http://www.cypalleles.ki.se). Proposed functional consequences for EFV pharmacokinetics have been reported for *CYP2B6* (15), *CYP2A6* and *CYP3A4|A5* (36). The study participants were categorized into genotypic groups according to the number of functional alleles (Table 1B). The simplest scoring scheme (Score A) assigned a value "2", "0" and "1" to, respectively, the fully functional reference (Hom-Ref), homozygous (Hom-LOF) heterozygous (Het-LOF) diminished/loss of function, and a value of "3" for *CYP2B6* gain of function alleles (Het-GOF). Two individuals having a single gene duplication of *CYP2A6* were assimilated to the Hom-Ref because of their small number. The classification was refined to distinguish between individuals with *CYP2B6* and *CYP2A6* loss/diminished function alleles to reflect the predicted level of activity from *in vitro* studies (Table 1 Scores B/C).

5.3.3.4. Pharmacokinetic Structural Model

EFV pharmacokinetics were characterized using a one-compartment model, as assessed previously (2). Since EFV is only administered orally, CL and *V* represent apparent values.

5.3.3.5. Covariate Model

The analyses of the covariate effects on CL were divided into three main sections that assessed (a)- the influence of demographic variables and concomitant medications (b)- the impact of *CYP2B6*, *CYP2A6, CYP3A4* and *CYP3A5* alleles based on univariate analyses and (c)- the joint effect of *CYP2B6* and *CYP2A6, CYP3A4* and *CYP3A5* alleles in multivariate analyses.

(a) Demographic Analyses

The typical value of CL was modeled to depend linearly on a covariate *X* (ie body weight, centered on the mean; categorical covariates coded as 0/1) as shown in the equation: $CL = \theta_a \cdot (1 + \theta_b \cdot X)$, where θ_a is the average estimate and θ_b is the relative deviation (positive or negative) from average attributed to the covariate *X*. The available demographic covariates were sex, ethnicity, age, body weight and height; few co-medications were recorded and were principally composed of other antiretroviral drugs and known *CYP* inducers or inhibitors (Table 2).

Table 2: Demographic and genetic characteristic of the population study

Characteristic	Value	% Of study population
Sex (No.)		
Men	124	73
Women	45	27
Age (y)		
Median (Range)	47 (30-73)	-
Body weight (kg)		
Median (Range)	77.5 (44-101)	-
Height (cm)		
Median (Range)	179 (153-193)	-
Ethnicity (No.)		
White	142	83
Black	16	10
Hispanic	6	4
Asian	5	3
PIs (No.)		
Ritonavir	20	13
Saquinavir	4	3
Amprenavir	0	0
Lopinavir	15	9
Atazanavir	18	11
NRTIs (No.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Abacavir	0	0
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (No.)		
Enfuvirtide	4	2
CYP P450 inducers (No.)	2	1
CYP P450 inhibitors (No.)	4	2
***CYP2B6* genetic polymorphism** (No.)		
Hom Ref	75	44
Het LOF	53	33
Hom LOF	23	14
Het GOF	16	9
Het LOF/Het GOF[†]	2	1
***CYP2A6* genetic polymorphism** (No.)		
Hom Ref	99	61
Het LOF	55	30
Hom LOF	13	8
Het GOF	2	1
***CYP3A4* genetic polymorphism** (No.)		
Hom Ref	138	82
Het LOF *1B	24	14
Hom LOF *1B	7	4
***CYP3A4* genetic polymorphism** (No.)		
Hom Ref	118	70
Het LOF_rs4646437	41	24
Hom LOF_rs4646437	10	6
***CY3A5* genetic polymorphism** (No.)		
Hom Ref	5	3
Het LOF	30	18
Hom LOF	134	79

Ref=reference allele, Het=heterozygous Hom= homozygous, LOF= loss of function, GOF= gain of function, NRT'sI=nucleoside reverse transcriptase inhibitors, NNRTI's= non-nucleoside reverse transcriptase inhibitors, PI's=Protease inhibitors.

[†] Two individuals are LOF/GOF for *CYP2B6* and were considered as Het LOF.

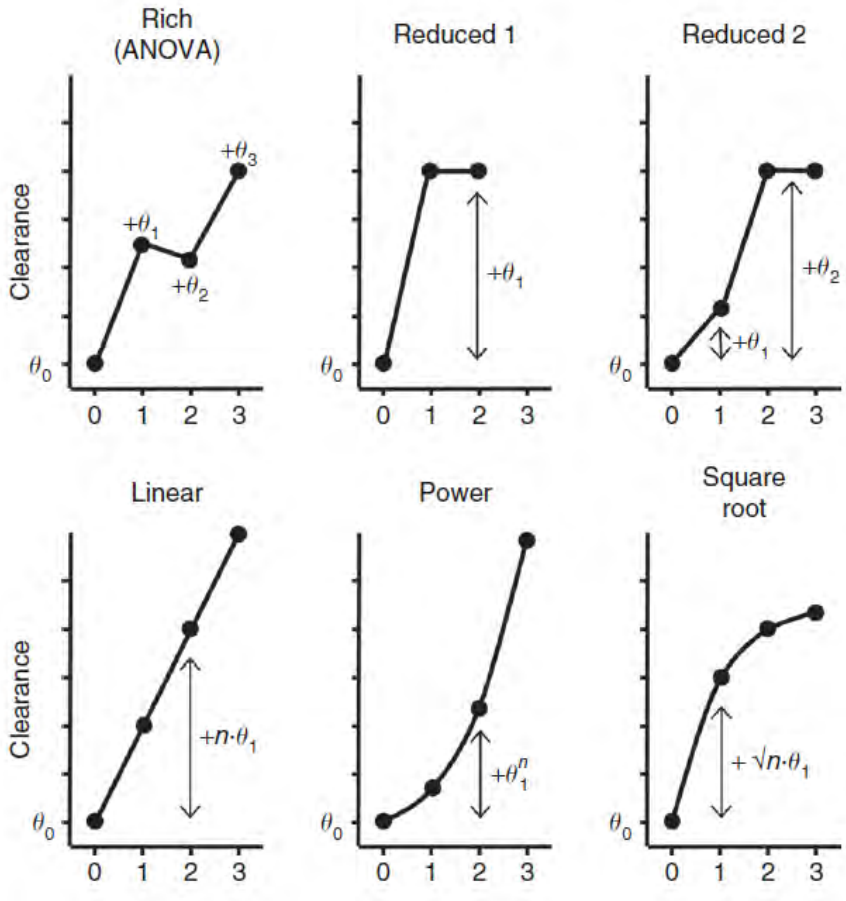

Figure 1: Different parameter models tested to describe the level of the oral clearance (Y-axis) as a function of the number of functional alleles of an enzyme (X-axis): 0 = Hom LOF, 1 = Het LOF, 2 = Hom Ref, 3 = Het GOF (a specific parameter for Het GOF, in grey, is only required with *CYP2B6*).

(b) Univariate genotype

In these analyses, each genotype was entered solely into the model. Several models relating CL with functional scores were tested using different ways (Figure 1) and compared with the richest possible model, which assigned a separate fixed effect to each score level as follows:

$$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3 \quad (1)$$

$$CL = CL_0 \cdot (1 + \theta_1 I_1) \cdot (1 + \theta_2 I_2) \cdot (1 + \theta_3 I_3) \quad (1a)$$

where CL_0 is the typical value of clearance in Hom-LOF individuals (Hom-Ref for *CYP3A5*), I_i is an indicator variable that takes the value of 1 if a individual carries the i^{th} genotypic score (i.e I_1: Het-LOF, I_2: Hom-Ref and I_3: Het-GOF) and 0 otherwise, and the θ_i are the absolute or fractional (eq.1/1a) change in CL relative to the Hom-LOF group. The impact of functional alleles on EFV CL was further explored to distinguish the

difference between the genotypic groups using two reduced models, in which the same genotyping group was assigned to Hom-Ref and Het-LOF or to Hom-Ref and Het-GOF carriers (Reduced 1 and 2, Figure 1). Competing models tried to account for a gene effect as a function of the number of functional alleles (Table1 Score A), using linear and power relationships with either additive or proportional (not shown) impact using the following models:

$$CL = CL_0 + \theta_1 \cdot n \quad (2)$$

$$CL = CL_0 + \theta_1^{\,n} \quad (3)$$

$$CL = CL_0 + \theta_1 \sqrt{n} \quad (4)$$

where n =1, 2 or 3 represents the functional score and θ_1 the average contribution per active allele above Hom-LOF CL (CL_0). The alternative activity scores B/C for *CYP2B6* and *CYP2A6* were explored using parameter models 2/3/4 above and compared with an extension of the rich model (eq.1).

(c) Gene-gene interaction analyses

The joint influence of functional alleles on EFV CL was first tested using pair-wise conjunction of *CYP2B6* with the other *CYP* alleles, to finally build up the model including all influent genetic variants. The investigation of the joint influence of *CYP2B6* and *CYP2A6* alleles is shown as an example. The richest model that served as reference for the evaluation of reduced competing models was:

$$CL = CL_0 + \theta_{01}I_{01} + \theta_{02}I_{02} + \theta_{10}I_{10} + \theta_{11}I_{11} + \theta_{12}I_{12} + \theta_{20}I_{20} + \theta_{21}I_{21} + \theta_{22}I_{22} + \theta_{30}I_{30} + \theta_{31}I_{31} + \theta_{32}I_{32} \quad (5)$$

here CL_0 is Hom-LOF CL for both genes and I_{ij} is an indicator variable that takes the value of 1 for the *CYP2B6* i^{th}/*CYP2A6* j^{th} genotype carrier and is "0" otherwise, and each θ_{ij} estimate the absolute change in CL among the different genotypic groups. The same model was parameterized for relative changes (not shown). The following competing models were evaluated:

$$CL = CL_0 + \theta_{0\_0} \cdot \sqrt{q} + (\theta_1 I_1 + \theta_{1\_1} \cdot \sqrt{q}) + (\theta_2 I_2 + \theta_{2\_2} \cdot \sqrt{q}) + (\theta_3 I_3 + \theta_{3\_3} \cdot \sqrt{q}) \quad (6)$$

$$CL = CL_0 + (\theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3) + \theta_4 \cdot \sqrt{q} \quad (7)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} \quad (8)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot (p \cdot q) \quad (9)$$

where *p* indicates the functional score for *CYP2B6* and *q* the score for *CYP2A6*. In eq.6, the contribution of *CYP2A6* ($\theta_{0\_0},..,\theta_{3\_3}$) is investigated on *CYP2B6* stratified by genotypic groups, in eq.7 the influence of *CYP2A6* is characterized using a single fixed effect parameter θ_4 across all *CYP2B6* genotypes, and in eq.8 square root functions are integrated for both genes; finally, an interaction term was allowed to further check for some non-additive interaction between *CYP* variants (eq.9). All the above models used either additive or proportional (not shown) effects.

All significant allelic groups were integrated into a final model, where the contribution of each genotypic group was estimated using a generalization of eq. 6/7/8, to be finally formulated using the following additive or proportional (not shown) relationships:

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot \sqrt{r} + \theta_4 \cdot \sqrt{s} + \theta_5 \cdot \sqrt{t} \quad (10)$$

where CL_0 is Hom-LOF CL for all genes and θ_i estimate the absolute or fractional change in CL as a function of score A for different combinations of *CYP2B6* (*p*), *CYP2A6* (*q*), *CYP3A4*_rs4646437 (*r*), *CYP3A4*1B* (*s*) and *CYP3A5* (*t*) alleles.

5.3.3.6. Variance Model

The individual clearance values were modeled assuming a log-normal distribution (mean zero and variance Ω). A proportional error model (mean zero and variance σ^2) was used for the description of intra-individual variability.

5.3.3.7. Parameter Estimation and Selection

NONMEM® (version VI, NM-TRAN version II) was used with FOCE INTERACTION to fit the models (35). As goodness of fit statistics, NONMEM® uses the objective function, which is approximately equal to minus twice the logarithm of the maximum likelihood. The likelihood ratio test, based on the reduction in objective function (ΔOF), was used to compare two models. A ΔOF (-2 log likelihood, approximate $\chi 2$ distribution) of 3.84, 5.99 and 7.81 points for 1, 2, or 3 additional parameters, respectively, was used to determine statistical significance ($p < 0.05$) between two models. The reliability of the results was checked on diagnostic goodness-of-fit plots, along with the measure of the standard errors. The identification of potential outlier values resulting from compliance issues or inadequacy in self-reporting information was explored by a sensitivity analysis. Except for one individual who had a concentration of 59'400 ng/ml that was at first excluded to prevent single outlier effect and integrated at the end, all data were considered reliable. Simulations based on the final pharmacokinetic estimates were

performed with NONMEM® using 1'000 individuals to calculate the 90% prediction intervals. The concentrations encompassing the 5th to 95th percentile at each time point were retrieved to construct the intervals. Further simulations in 1'000 individuals at various dosage regimens (200, 400, 600 and 800 mg) for a series of genotype combinations were performed to suggest doses ensuring trough levels to be comprised within the 1-4 mg/ml therapeutic interval in 90% of the individuals. The figures were generated with GraphPad Prism (Version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

5.3.4. Results

In total, 393 plasma samples were collected in 169 individuals. Concentration measurements ranged between 100 and 59'400 ng/ml. A 1-compartment model with first order absorption from the gastrointestinal tract fitted the data appropriately. Average clearance (CL) was 11.3 L/h with an interindividual variability of 65%, volume of distribution (V) was 388 L and the absorption constant (*ka*) 0.62 h^{-1}. The assignment of interindividual variability on either *V* or *ka* did not improve the fit (ΔOF=0.0).

5.3.4.1. Demographic analyses

Body weight and Black ethnicity influenced CL, as well as gender, age and height to a lesser extent. No co-medications were shown to influence EFV pharmacokinetics significantly. A multivariable combination of demographic factors revealed that body weight accounted for the effect of height, age and gender, while explaining 3% of CL variability, and was the only demographic factor influencing CL outside Black ethnicity, which remained statistically significant beyond body weight and reduced variability in CL by another 3%.

5.3.4.2. Univariate genotype analyses

The influence of *CYP2B6*, *CYP2A6*, *CYP3A4/AA5* functional alleles on EFV CL was first tested in single-gene analyses, in which the allelic variants (Hom-LOF, Het-LOF and Hom-Ref plus Het-GOF for *CYP2B6*) were entered into the model as covariates that partitioned individuals based on their genetic constitution.

Genetic variation of *CYP2B6* had by far the most salient impact on CL. Several competing models were tested, as depicted in Figure 1. The richest possible model, which assigned a separate fixed effect for each *CYP2B6* allelic variants (eq.1/1a) improved markedly the fit and explained 31% out of the 65% interindividual variability

on CL. Average CL was 2.8 L/h in the Hom-LOF group and 10.8 L/h, 13.3 L/h and 18.8 L/h in individuals carrying Het-LOF, Hom-Ref and Het-GOF alleles, respectively. A series of reduced models showed CL to be statistically different among all *CYP2B6* groups (ΔOF=+9 and ΔOF=+10 for Model Reduced 1 and 2). Competing simplified models tried to estimate CL as a function of the number of functional alleles, as defined by the activity score A (Table 1) and were compared to the richest model (eq.1/1a); the use of a linear model (eq.2) or a power function model (eq.3) did not fit the data appropriately (ΔOF=+18.5, +58.8, respectively). As interim explorations showed CL to be modestly reduced by about 25% in Het-LOF carriers but cut down by 75% in Hom-LOF individuals, a square root function model achieved the best fit using either an additive (eq.4) or proportional model (ΔOF=-167.7). The recourse to alternative activity scores B for *CYP2B6*, allowing for the distinction between loss/decrease of function alleles, did not better characterize the genotype-phenotypes relationships using any of eq.2/3/4 (ΔOF>+8.2).

The assignment of *CYP2A6* allelic variants on CL using the richest model (eq.1/1a) improved the fits (ΔOF=-7.9) and decreased by 1% the overall variability on CL. Average CL was 7.0 L/h, 10.8 L/h and 12.1 L/h in Hom-LOF, Het-LOF and Hom-Ref individuals, respectively. The difference in CL between Hom-Ref and Het-LOF individuals was not significant (ΔOF=-1.0). The description of the relationship between CL and the functional score using either linear (eq.2) or power models (eq.3) did not fit the data adequately compared to the richest model (ΔOF>+6.8), whereas it was again best characterized using a square root function (eq.4) that provided almost identical fit as the rich model (ΔOF= -7.8). Neither here did the model integrating partial activity levels (Score B/C) improve data description (ΔOF=-0.4 for Score B and -0.1 for Score C).

The impact of *CYP3A4* on EFV CL was tested using two alleles associated with changes in function, *CYP3A4*1B* and *CYP3A4*_rs4646437. The rich model (eq.1/1a) showed that both alleles influenced CL to a significant extent (ΔOF=-25.4 for *CYP3A4*_rs4646437, -10.4 for *CYP3A4*1B*). CL in Hom-LOF, Het-LOF and Hom-Ref carriers were, respectively, 5.1 L/h, 10.3 L/h and 11.9 L/h for *CYP3A4*1B* and 3.7 L/h, 10.7 L/h and 12.3 L/h for *CYP3A4*_rs4646437 alleles. Interindividual variability in CL dropped from 65% to respectively 62% and 59% after inclusion of *CYP3A4*1B* and *CYP3A4*_rs4646437 alleles in the model. Difference in CL between Hom-Ref and Het-LOF individuals were not significant for both **1B* and rs4646437 (ΔOF=-1.4 for *CYP3A4*_rs4646437 and=-0.9 for *CYP3A4*1B*). Compared to the richest model, linear and power function models (eq.2/3)

did not fit the data adequately (ΔOF>+8.0), while square root models (eq.4) described the data at best (ΔOF=-10.3 for *CYP3A4*1B* and -24.8 for *CYP3A4*_rs4646437).

The influence of *CYP3A5* functional alleles on CL showed a small but significant effect on CL using the rich model (eq.1/1a, ΔOF=-8.1), with a remaining 64% interindividual variability. The difference in CL between Het-LOF and Hom-Ref alleles was not significant (ΔOF=-0.0). None of the above models (eq.2/3) could characterize the relationship between CL and *CYP3A5* allele variants better than the square root one (eq.4, ΔOF=-8.2).

The effect of Black ethnicity remained a statistically influencing covariate on CL in addition to genetic variation, causing an additional 25% to 40% decrease in CL when associated with functional alleles (ΔOF compared to *CYP2B6*=-8.1, *CYP2A6*=-9.9, *CYP3A4*1B*=-4.0 and *CYP3A5*=-7.8), except for *CYP3A4*_rs4646437 (ΔOF=-0.4), which was present in most of the Black individuals, thus limiting the power to detect any association.

5.3.4.3. Gene-gene interaction analyses

The joint influence of functional alleles on EFV CL was first tested through the conjunction of *CYP2B6* with each other *CYP* alleles in dual gene models, to finally build up the model including all influent genetic variables. The richest model (eq.5) characterizing the joint influence of *CYP2B6* and *CYP2A6* using a fixed effect parameter for each allelic combination suggested an additional contribution of *CYP2A6* in EFV elimination (ΔOF=-20 compared to the final model for *CYP2B6* eq.4). Competitive models were developed based on a functional score A (Table 1) using square root function models. The models evaluating the contribution of *CYP2A6* variation either on each *CYP2B6* genotypic groups separately (eq.6) or using a single parameter estimate across all *CYP2B6* allelic variants (eq.7) fitted the data with similar adequacy (ΔOF>-16), resulting in an absolute increase in CL of 1.2 L/h and 1.7 L/h in *CYP2A6* Het-LOF and Hom-Ref individuals, respectively, compared to Hom-LOF carriers. The contribution of *CYP2A6* functional alleles was more prominent in *CYP2B6* Hom-LOF carriers, in whom the relative change in CL was estimated to be 44% per active allele compared to an 11% change in Hom-Ref individuals. The joint gene influence could be further described by simply adding *CYP2B6* and *CYP2A6* square root functions (eq.8 ΔOF=-12). This model was not considered statistically different from previous models (eq.6/7), considering the reduced degrees of freedom and the loss of fit from the richest possible

model (eq.5, ΔOF=+8). The introduction of a single interaction factor (eq.9) to evaluate some hyper- or hypo-additive trend was not significant (ΔOF=+3).

The same paradigm was successfully applied to characterize the other gene-gene interactions. The rich model (eq.5) characterizing the joint influence of *CYP2B6* with *CYP3A4*1B* or *CYP3A4* rs4646437 alleles suggested an additive conjunction of both genes on EFV CL (ΔOF=-14 for **1B*, -25 for rs4646437*).* Reduced models integrating square root functions of *CYP3A4* activity scores on *CYP2B6* stratified by allelic variation (eq.6) described the data appropriately (ΔOF=-13 for **1B,* -18 for rs_4646437)*,* and no deterioration of the fit was observed when allowing a single parameter estimate (eq.7) for the effect of *CYP3A4* across all *CYP2B6* allelic variants (ΔOF= +1.0 for **1B,* 0.0 for rs_4646437 compared to eq.6)*.* The additive contribution of *CYP3A4* on CL was 1.1 and 1.5 L/h in **1B* Het-LOF and Hom-Ref carriers, respectively, and 1.4 and 1.9 L/h in rs_4646437 Het-LOF and Hom-Ref carriers, respectively. CL increased by 40% *(*1B)* and 48% (rs_4646437) per active allele of *CYP2B6* and *CYP3A4* compared to respective Hom-LOF. Further characterization of the joint contribution of *CYP2B6* and *CYP3A4* on EFV CL using a mere addition of square root functions (eq.8) fitted appropriately the data, the loss of fit compared to previous models (eq.5/6/7) being not significant (ΔOF=-9.0 for **1B*, -18 for rs_4646437). No factor accounting for more than an additive interaction was observed (ΔOF=0.0).

The joint assignment of *CYP2B6* and *CYP3A5* on CL improved the fit as well compared to *CYP2B6* solely (eq.5, ΔOF=-16). The successive nesting of models using functional scores followed successfully the same paradigm as previously described for other *CYPs*. The influence of *CYP3A5* on *CYP2B6* was appropriately described using eq.6 or eq.7 (ΔOF>-13) or as a square root additive model (eq.8, ΔOF>-10). Again, an interaction between both genes was not significant (ΔOF=0.0).

A final joint model characterizing the cumulative influence of all genetic variants on EFV CL was tested based on the two-by-to combinations of genetic effects. The richest model that integrated the effects of all CYPs alleles using a generalization of eq.6/7 improved the fit (ΔOF>-29 compared to *CYP2B6* solely), but the impact of *CYP3A4*1B* and *CYP3A5* did not remain significant (ΔOF=0.0 compared to the rich model without those two alleles). The final model employed a single parameter estimate to quantify the influence of each *CYP2B6*, *CYP2A6* and *CYP3A4*_rs4646437 alleles using additive square root functions, which fitted the data appropriately (eq.10, ΔOF=-24). All models specified with proportional rather than additive effects gave very similar results (ΔOF<-0.5). In addition to genetic influences on CL, the influence of body weight remained the

only significant demographic covariate (∆OF=19.2), while any ethnic influence vanished completely. The final model estimated an average CL of 1.3 L/h in individuals carrying Hom-LOF *CYP2B6*, *CYP2A6* and *CYP3A4*_rs4646437*,* which increased by 7.3 L/h, 0.7 L/h and 1.03 L/h for the first active allele of those genes, respectively, and by another 0.41 times those factors for the second allele, (where $0.41 = \sqrt{2} - 1$), with an additional 70% increase in CL on body weight doubling. A summary of model building procedure is presented in Table 3 and the final population estimates in Table 4. A plot of the model-predicted concentration profile stratified by *CYP2B6* is shown in Figure 2. Figure 3 represents the individual posthoc CL estimates with population average predictions for the different allelic combinations encountered.

Based on our final model, simulations show that, with the standard regimen of 600 mg of EFV daily, average trough concentrations are 1.19 mg/L (90% prediction interval: 0.6–2.35), 1.6 (0.8–2.5) and 8.1 mg/L (4.5–14.5) in *CYP2B6* Hom-Ref, Het-LOF and Hom-LOF carriers, respectively and 0.5 mg/L (0.2-1.0) in *CYP2B6* Het-GOF carriers. Taking into account the interindividual variability leads to the suggestion that most *CYP2B6* Hom-LOF individuals will exhibit concentrations exceeding the 1-4 mg/L range that is generally considered acceptable, and most individuals carrying a Het-GOF will have concentrations < 1 mg/L. Predicted concentrations of 2.7 mg/ml (1.5–4.9) would be expected at a dosage regimen of 200 mg/day in *CYP2B6* Hom-LOF carriers. However, individuals having Hom-LOF of *CYP2A6*/*CYP3A4*_rs3434367 and *CYP2B6* will be exposed to considerably higher drug levels, yielding average predicted concentrations of 6.2 mg/ml (3.5–11.0) with a regimen of 200 mg/day.

Table 3: Summary of the key models used to examine the influence of demographic and genetic covariates on EFV clearance.

Step 1	Demographic model	Model	θ_0	θ_1	θ_2	θ_3	ΔOF	P
	Body weight (BW)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot BW)$	11.5	1.2			−25.6	**
	Height	$CL = \theta_0 \cdot (1 + \theta_1 \cdot Hgt)$	11.2	2.3			−4.2	*
	Age	$CL = \theta_0 \cdot (1 + \theta_1 \cdot age)$	12	0.7			−5.7	*
	Sex (M = 0, F = 1)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot sex)$	12	0.2			−4.1	*
	Race	$CL = \theta_0(1 - q) + \theta_1 \cdot q$						
	Black ($q = 1$) vs. others ($q = 0$)		11.8	6.25			−13.2	**
	White ($q = 1$) vs. others ($q = 0$)		12	7.1			−7.7	**
	Hispanic ($q = 1$) vs. others ($q = 0$)		15.1	11.2			−0.9	NS
	Asian ($q = 1$) vs. others ($q = 0$)		11.3	9.8			−0.13	NS
	Other ARV							
	Ritonavir (RTV)	$\theta_0 \cdot (1 + \theta_1 \cdot RTV)$	11.3	0.01			0.2	NS
	Zidovudine (AZT)	$\theta_0 \cdot (1 + \theta_1 \cdot AZT)$	10.4	0.12			−1.1	NS
	Lamivudine	$\theta_0 \cdot (1 + \theta_1 \cdot 3TC)$	10.6	0.09			−0.6	NS
	NRTI in general	$\theta_0 \cdot (1 + \theta_1 \cdot NRTI)$	8.6	0.3			−1.8	NS
	PI in general	$\theta_0 \cdot (1 + \theta_1 \cdot PI)$	11.3	0.01			0.6	NS
Step 2	**Genotype-variant analysis**	**Model**	CL_0	θ_1	θ_2	θ_3	ΔOF	P
CYP2B6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3$	2.8	10.8	13.3	18.8	−171	**
Reduced 1	I_1: Het-LOF or Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_3 I_3$	2.8	12.2	—	18.9	−162	**,[a]
Reduced 2	I_1: Het-LOF, I_2: Hom-Ref or GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	2.8	10.8	14.2		−161	**,[a]
Eq. 2	$n = 0, 1, 2, 3$	$CL = CL_0 + \theta_1 \cdot n$	3.11	5.99			−152	**,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	5.03	2.9			−76	**,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	2.8	7.8			−168	NS[a]
CYP2A6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	7.0	10.8	12.1		−7.9	*
Reduced 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	7.0	11.7			−6.9	NS[a]
Eq. 2	$n = 0, 1, 2$	$CL = CL_0 + \theta_1 \cdot n$	7.75	2.31			−6.8	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.8	2.11			−5.3	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	7.0	3.63			−7.8	NS[a]
CYP3A4 rs4646437								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	3.7	10.7	12.3		−25.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	3.7	11.9			−24	NS[a]
Eq. 2	$n = 0, 1, 2$	$CL = CL_0 + \theta_1 \cdot n$	4.84	4.06			−20	*,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.2	2.5			−12.9	*.a
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	3.89	6.1			−24.8	NS[a]
*CYP3A4*1B*								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	5.1	10.3	11.9		−10.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	5.1	11.7			−9.5	NS[a]
Eq. 2	$n = 0, 1, 2$	$CL = CL_0 + \theta_1 \cdot n$	6	3.6			−9.0	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.4	2.3			−7.0	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	5.2	4.8			−10.4	NS[a]
CYP3A5								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-LOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	4.4	10.3	11.8		−8.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-LOF	$CL = CL_0 + \theta_1 I_1$	4.4	11.5			−7.4	NS[a]
Eq. 2	$n = 0, 1, 2$	$CL = CL_0 + \theta_1 \cdot n$	6.15	2.96			−6.4	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.0	2.22			−4.8	*,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	4.7	5.2			−8.1	NS[a]

Step 3	Gene–gene interaction analysis	*CYP* contribution	CL_0	θ_1	θ_2	θ_3	θ_4	ΔOF[a]
Eq. 5[b]	*CYP2B6/CYP2A6*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.8	2.59 7.42 9.74 14.6	3.15 11.6 11.9 19.6	10.9 14.3 18.8		−16
Eq. 6	*CYP2B6/CYP2A6*	*CYP2B6* *CYP2A6* $\cdot\sqrt{q}$	1.75	8.15 0.95	2.42	9.21 3.44	19.3 2.49	−17
Eq. 7	*CYP2B6/CYP2A6*	*CYP2B6* *CYP2A6* $\cdot\sqrt{q}$		2.02 1.21	7.7	9.45	13.6	−16
Eq. 8	*CYP2B6/CYP2A6*	*CYP2B6* $\cdot\sqrt{p}$ *CYP2A6* $\cdot\sqrt{q}$	1.5	7.7 1.2				−12
Eq. 5[b]	*CYP2B6/CYP3A4 _rs4646437*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.66	2.46 10.5 13.3 16.3	3.9 11 12.3 17.0	 10.6 13.5 19.3		−25
Eq. 6	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6* *CYP3A4 _rs* $\cdot\sqrt{r}$		1.6 1.4	10.7 0.1	11.2 1.6	15.3 2.6	−22
Eq. 7	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6* *CYP3A4 _rs* $\cdot\sqrt{r}$		1.62 1.36	8.99	11.5	16.9	−22
Eq. 8	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6* $\cdot\sqrt{p}$ *CYP3A4 _rs* $\cdot\sqrt{r}$	1.62	7.3 1.34				−18
Eq. 5[b]	*CYP2B6/CYP3A4*1B*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.69	2.33 10.6 13.4 16.4	3.23 10.3 11.7 17.2	 10.8 13.6 19.4		−14
Eq. 6	*CYP2B6/CYP3A4*1B*	*CYP2B6* *CYP3A4*1B* $\cdot\sqrt{s}$		1.64 1.1	10.7 0.4	11.2 1.2	15.3 2.7	−13
Eq. 7	*CYP2B6/CYP3A4*1B*	*CYP2B6* *CYP3A4*1B* $\cdot\sqrt{s}$		1.62 1.1	8.99	11.5	16.9	−12
Eq. 8	*CYP2B6/CYP3A4*1B*	*CYP2B6* $\cdot\sqrt{p}$ *CYP3A4*1B* $\cdot\sqrt{s}$	1.65	7.6 1.1				−9
Eq. 5[b]	*CYP2B6/CYP3A5*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.45	2.22 6.0 13.4 16.5	3.25 11.6 11.9 17.0	10.6 13.6 19.2		−16
Eq. 6	*CYP2B6/CYP3A5*	*CYP2B6* *CYP3A5* $\cdot\sqrt{t}$		1.38 1.1	10.7 0.1	11 1.8	15 2.4	−14
Eq. 7	*CYP2B6/CYP3A5*	*CYP2B6* *CYP3A5* $\cdot\sqrt{t}$		1.4 1.22	9.1	11.7	17.1	−13
Eq. 8	*CYP2B6/CYP3A5*	*CYP2B6* $\cdot\sqrt{p}$ *CYP3A5* $\cdot\sqrt{t}$	1.4	7.6 1.22				−10

PI= protease inhibitors, NRTI=non nucleoside/nucleotide reverse transcriptase inhibitors, ARV=antiretroviral medication, n, p, q, r, s = numbers of functional alleles (0 = Hom LOF, 1 = het LOF, 2 = Hom Ref, 3 = Het GOF), ΔOF: difference in the objective function, compared to the final model structural model, ΔOF: difference in the objective function compared to the model including CYP2B6 solely.

[a] differences in objective function compared to the rich model (Eq. 1)

[b] I_{xy} represents the number of functional alleles for the x/y cytochromes. For clarity, θ have been numbered from 1 to 4. *= P<0.05, ** = P<0.01, NS = not statistically significant.

Table 4: Final population pharmacokinetic parameter estimates of EFV

Parameter[a]	Population mean		Interindividual variability	
	Estimate	**SE (%)[c]**	**Estimate (%)[b]**	**SE (%)[d]**
CL/F (L/h)[e]	1.3	18	27.8	43.8
θ_{2B6}[f]	7.3	7		
θ_{2A6}[f]	0.7	36		
$\theta_{3A4\_rs4646437}$[f]	1.03	27		
θ_{BW}[g]	0.7	42		
Vd/F (L)	332	16		
Ka (h^{-1})	0.6	38		
σ (C.V %)[h]	30.8	44.2		

[a] CL/F, mean apparent clearance; V/F, mean apparent volume of distribution; ka, mean absorption rate constant; F, bioavailability.
[b] Estimate of variability is expressed as CV (%).
[c] Standard errors of the estimates (SE),defined as SE/estimate and expressed as percentages.
[d] Standard errors of the coefficient of variation, taken as $\sqrt{\mathrm{SE}/\mathrm{Estimate}}$ and expressed as percentage.
[e] *CL* value in patients with Hom LOF for *CYP2B6*, *CYP2A6* and *CYP3A4*_rs4646437
[f] Contribution of *CYP2B6*, *CYP2A6* and *CYP3A4*_rs4646437 to EFV *CL* multiplied by $\sqrt{n}$ where n = 0,1,2,3 for *CYP2A6,* n=0, 1, 2 for *CYP2A6* and *CYP3A4*_rs4646437 (see text).
[g] Relative influence of body weight on EFV clearance (see text)
[h] Residual intrapatient variability, expressed as a CV (%)

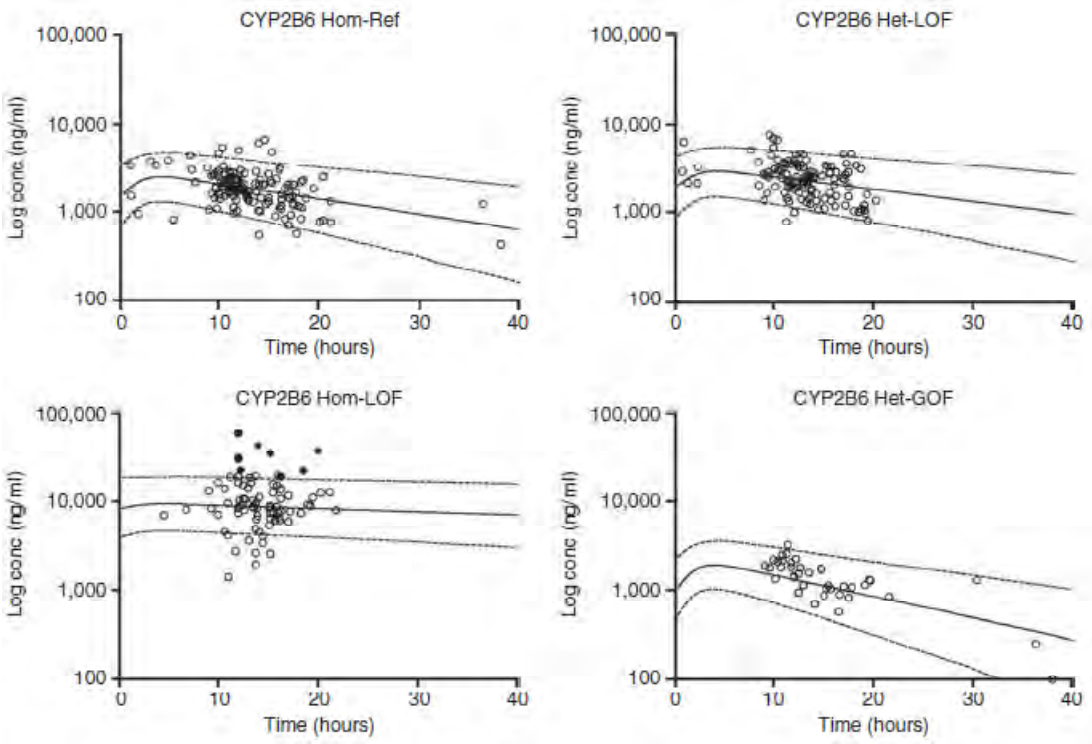

Figure 2: EFV plasma concentrations (n=393) in 169 HIV-1 individuals (open circles) according to *CYP2B6* polymorphism with population predictions of the corresponding genotype represented by black lines and 90 % prediction interval (grey dotted lines). Left low panel: full dark circles represent concentrations in individuals Hom LOF for CYP2B6, CYP2A6 and CYP3A4_rs46464337 while full dark diamonds represent concentrations in individuals Hom LOF for CYP2B6 and Het LOF for CYP3A4_rs4646437.

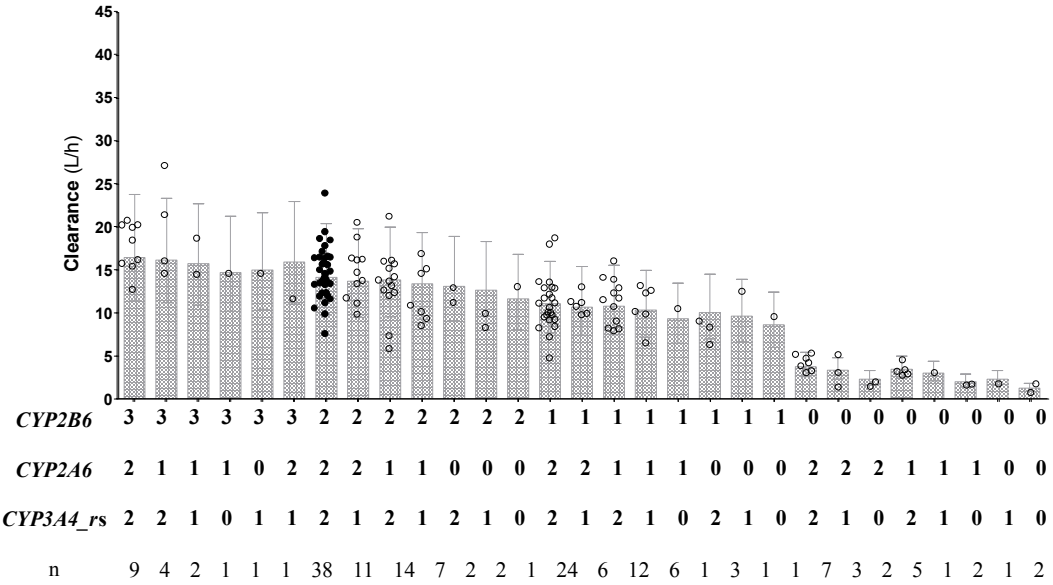

Figure 3: Individual predicted Bayesian clearances (open circles) and average predicted clearance (bars) with 90% population prediction interval for each *CYP2B6, CYP2A6* & *CYP3A4*_rs4646437 allelic combinations (according to Score A categorization: 0: Hom LOF/DOF, 1 Het LOF/DOF, 2 Hom Ref, 3 Het GOF see text); n = number of individuals carrying the allelic combination (n= 0 for 8 combinations not represented).

5.3.5. Discussion

This study was able to characterize and quantify for the first time the conjugated effects of major and minor metabolic pathways and their genetic variants on EFV pharmacokinetics in a population of HIV-1 infected patients. EFV average CL and variability are in the range of values reported previously (1-3). Among non-genetic covariates, we observed an impact of body weight on CL, as reported by others (16-20). No differences could be observed between male and female subjects, while conflicting results exist in the literature (20-23). The well-known influence of Black ethnicity, which has been associated with *CYP2B6* and *CYP3A4* heterogeneity (24;25), could be mostly explained by the joint influence of *CYP2B6*, *CYP2A6* and *CYP3A4* variations, whereas its influence remained discernible when these *CYP* alleles were considered separately. Due to a limited number of Hispanic (n=6) and Asian (n=5) individuals in our study, no clear effect on EFV could be attributed to these ethnic groups, although an influence has been reported previously (1;3;10;20). Among genetic covariates, *CYP2B6* allelic variation accounted for most of the interindividual differences in EFV CL. The *CYP2A6* and *CYP3A4/A5* accessory pathways appeared to influence EFV elimination independently from *CYP2B6*. Among those, the overall impact of *CYP3A4*_rs4646437 was the largest and accounted for 6% of CL variability. The unexpected lower CL in *CYP3A5* Hom-Ref compared to Hom-LOF carriers can be explained by the linked Hom/Het-LOF of *CYP2B6* and *CYP3A4*_rs4343437 observed in most individuals. This hypothesis is further confirmed by the lack of effect of *CYP3A5* when the influence of all cytochromes is assessed in a joint analysis. When information on *CYP2B6* was included along with *CYP2A6* and *CYP3A4* in joint analyses, an additive effect of accessory pathways was still present both on fully functional *CYP2B6* and in presence of reduced or gain of function alleles. The compensation ensured by these *CYP* alleles was however small (1-2 L/h) and therefore more discernible on *CYP2B6* Hom-LOF. An evaluation of *CYPs* genetic variation according to an activity score, as recently proposed by Gaedigk et al. (26) for *CYP2D6*, enabled us to quantify genetic influences of all alleles based on the same paradigm. A model of remarkable parsimony, which included only one parameter per allele and required no interaction term, could capture the non-linear relationship between CL and the different genotypic groups. The use of such square root relationships in gene-dose-effects was not described for other *CYP* isoenzymes, in particular for the non-inducible *CYP2D6* (24) that rather shows linear effects (27). This phenomenon suggests adaptive mechanisms that might be explained by the up-regulation of extensive metabolizer alleles in response to concentration increase,

possibly trough the activation of nuclear receptors (28;29). It is noteworthy that a similar pattern has already been reported for *CYP2B6* not only with EFV (30) but also with S-methadone (31). The use of different functional score classifications to implement partial activity levels failed to improve the model in comparison with the traditional classification. The mechanisms by which allelic variants express a loss/diminished function could therefore not be translated in a "semi quantitative gene-dose system", as described by Steimer et al. (32) for amitriptyline and nortriptyline.

The cumulative influence of *CYP2B6*, *CYP2A6* and *CYP3A4*_rs4646437 allelic variants on CL implies a critical 90% decrease of EFV elimination in triple Hom-LOF individuals, in whom CL appeared reduced to 1.3 L/h, compared to the estimated 12.9 L/h in triple Hom-Ref individuals. Of note, the only individual exhibited extremely high EFV concentrations (33) was found to be triple Hom-LOF, which emphasizes the importance of accessory pathways in EFV elimination. The interindividual variability in CL dropped from 65% to 27% in the final model, with *CYP2B6* genetic variants accounting for 31% of this variability and another 7% being explained by *CYP2A6, CYP3A4*_rs4646437 and body weight variations. The remaining variability might be attributed to adherence issues (34) or to variation in UGT metabolic pathways.

In conclusion, functional alleles of *CYP2B6* accounted for the majority of EFV interindividual variability. Genetic variation in EFV accessory metabolic pathways demonstrated their importance in EFV pharmacokinetics in addition to *CYP2B6*, in particular in individuals with limited *CYP2B6* function. Dosage reduction to 200 mg per day in *CYP2B6* impaired function is required to ensure drug level within the therapeutic range. The expression of pharmacogenetic influences on EFV elimination could be characterized using a single common paradigm across all *CYPs*, which adds a contribution of each enzymatic pathway proportional to the square root of the number of functional alleles. Such a model predicts appropriately average CL for various allele combinations, and its mechanistic explanation warrants further investigation.

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Chapter 6:
Optimising ART for new drugs

New ARV drugs are continuously marketed to improve antiretroviral therapy for HIV-infected patients with limited or no remaining treatment options. Besides, validated analytical method are necessary for monitoring ARVs levels in new regimen combinations administered as salvage therapy to patients having frequently experienced treatment failure, and for whom exposure, tolerance and adherence assessments are critical.

Raltegravir, the first approved HIV integrase inhibitor, is probably the drug that attracted the most attention amongst the new molecules developed these recent years. Raltegravir was made available in Switzerland for treatment-experienced patients with multi-drug resistant strains of HIV-1. It has demonstrated a potent antiviral activity in patients with limited options. More recently, in treatment-naive patients, it has been associated with a much more rapid reduction in viral load than its competitors. Raltegravir is characterised by an extremely variable and unpredictable pharmacokinetics that may be influenced to food composition and intake, pH-dependant solubility, polymorphism of the UDP-glucuronosyltransferase 1A1 (its major metabolic pathway), and P-glycoprotein expression levels.

Given the high PK variability, impressive antiretroviral activity in general, and favourable safety profile at the recommended dose of 400 mg twice daily, relationships between plasma exposure and virological response or toxicity have been so far difficult established for raltegravir, suggesting that other markers than total concentrations may better reflect its antiviral activity.

6.1. Cell disposition of raltegravir and newer antiretrovirals in HIV-infected patients: high inter-individual variability in raltegravir cellular penetration

At present, no consistent PK/PD relationships could have been evidenced using raltegravir (RAL) concentration in plasma, suggesting that other markers reflecting RAL exposure may better predict its pharmacological activity in patients. RAL exerts its antiviral activity within the infected cells. However, there is at present limited information on the extent of RAL cellular exposure and the relationships between RAL levels measured *in vivo* in plasma and in PBMCs from HIV patients.

In collaboration with the HIV Unit at CHUV, an open-label, phase IV pharmacokinetic study has been initiated in order to determine, in HIV-positive patients, concentrations of raltegravir and newer antiretrovirals in plasma and in cells (PBMCs) over one dosing interval. Raltegravir total plasma concentrations were analysed by LC-MS/MS using the method described in Chapter 2.1., whereas cellular concentrations were determined with an adaptation of the same LC-MS/MS assay using our methodology successfully applied in a number of investigations on antiretroviral cellular concentrations (1,2)

Thus, if cell penetration appears to represent a key mechanism in the development of raltegravir activity, and conversely, of resistance in case of insufficient cellular disposition, it may open the way to further determinations or interventions aimed to optimise its therapeutic utilisation.

Related appendices: 6.1., 6.2., 6.3., 6.4.

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CELL DISPOSITION OF RALTEGRAVIR AND NEWER ANTIRETROVIRALS IN HIV-INFECTED PATIENTS: HIGH INTER-INDIVIDUAL VARIABILITY IN RALTEGRAVIR CELLULAR PENETRATION

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6.1.1. Abstract

6.1.1.1. Objectives

The site of pharmacological activity of raltegravir (RAL) is intracellular. Our aim was to determine the extent of RAL accumulation and whether RAL total plasma concentrations (C_{tot}) predict cellular concentrations (C_{cell}).

6.1.1.2. Design and Methods

Open-label, prospective, pharmacokinetic study on HIV-infected patients on a stable RAL-containing regimen. Plasma and peripheral blood mononuclear cells were simultaneously collected during a 12h- dosing interval after drug intake. C_{tot} and C_{cell} of RAL, darunavir (DRV), etravirine (ETV), maraviroc (MVC) and ritonavir (RTV) were measured by LC- MS/MS after protein precipitation. Longitudinal mixed effects analysis was applied to the C_{cell}/C_{tot} ratio.

6.1.1.3. Results

Ten HIV-infected patients were included. The geometric mean (GM) RAL total plasma maximum concentration (C_{max}), minimum concentration (C_{min}) and area under the time-concentration curve (AUC_{0-12}) were 1068 ng/ml, 40 ng/ml and 4171 ng*h/ml, respectively. GM RAL cellular C_{max}, C_{min} and AUC_{0-12} were 27.5 ng/ml, 2.7 ng/ml and 165 ng*h /ml. RAL C_{cell} corresponded to 5.3 % of C_{tot} measured simultaneously. Both concentrations fluctuate in parallel, with C_{cell}/C_{tot} ratios remaining stable for each patient without significant time-related trend over the dosing interval (CV_{intra}=83%), whereas inter-individual variability reaches 96%, explaining 62% of the total variability. The AUC_{cell}/AUC_{tot} GM ratios for RAL, DRV and ETV were 0.039, 0.14 and 1.55. RAL C_{cell}/C_{tot} ratios were not influenced by the presence of DRV/RTV nor ETV.

6.1.1.4. Conclusions

RAL C_{cell} are correlated with C_{tot} (r=0.86). RAL penetration in cells is overall low (ca 5% of plasma levels), with distinct RAL cellular accumulation varying by as much as 15-fold between patients. The importance of this finding in the context of resistance development to integrase inhibitors needs to be further investigated.

6.1.2. Introduction

Raltegravir (RAL) has demonstrated a potent antiviral activity with so far a favorable safety profile.[1-6] It is associated with a much more rapid reduction in viral load than other antiretroviral molecules in treatment-naive patients,[7] and has performed much better than anticipated, even with patients with limited options who have subtherapeutic RAL plasma levels.[8] Raltegravir is characterized by large intra-individual (122%) and inter-individual (212%) pharmacokinetics variabilities that may be related to food composition and intake, pH-dependent solubility (with higher solubility at increasing pH), polymorphism of the UDP-glucuronosyl-transferases (UGTs), and P-glycoprotein (P-gp) expression levels. Given the high pharmacokinetic variability and favorable safety profile at the recommended 400 mg BID dose, relationships between RAL plasma exposure and virological response or toxicity have been so far difficult to establish. No consistent pharmacokinetic/pharmacodynamic (PK/PD) relationships could have been evidenced so far using RAL concentration in plasma,[9] suggesting that other markers of RAL exposure may better predict its pharmacological activity in patients. RAL exerts its antiviral activity within the infected cells. However, there is at present very limited information on RAL penetration in cells, the extent of RAL cellular exposure and the relationships between RAL levels measured in vivo in plasma and in peripheral blood mononuclear cells (PBMCs) from HIV patients.

We therefore initiated a pilot prospective pharmacokinetic study to determine over one dosing interval (12h) RAL concentrations simultaneously in plasma and PBMCs in HIV-infected patients on stable RAL-containing regimens. Our aims were to assess whether RAL plasma levels can reflect cellular levels, and to determine the overall RAL cellular disposition, its intra- and inter-patient variability and its correlation with plasma concentrations. Moreover, cellular disposition of etravirine (ETV), maraviroc (MVC), darunavir (DRV) and ritonavir (RTV) were similarly determined in cells and in plasma from patients receiving those drugs in combination with RAL.

6.1.3. Methods

6.1.3.1. Study design

This was an open-label, prospective, pharmacokinetic study on HIV-infected patients on a stable salvage RAL-containing regimen. The study was performed in accordance with the Declaration of Helsinki and its amendments, and in compliance with the guidelines of Good Clinical Practice.

Patients were admitted to the hospital at 7.15 a.m., 45 minutes before the time of their morning dose of RAL. They received a standardized breakfast at 7.45 a.m. before drug intake, and standardized lunch and dinner at 12 a.m. and 19 p.m., respectively. For C_{tot} determination, 6 blood samples (5 ml; Monovettes® with EDTA-K, Sarstedt, Nümbrecht, Germany) were collected starting at pre-dose (time -0.5 h) and 1, 3, 6, 8 and 12 h after oral administration of the drugs (time 0 h). Additional 8 ml of blood samples (Vacutainer™ CPT tubes with sodium citrate, Becton Dickinson, Franklin Lakes, NJ, USA) were collected at -0.5, 3, 8 and 12 h post-dose for C_{cell} determination. Patients were also invited to report exact date and time of the last 3 RAL doses, and composition of the accompanying meal, and exact date and time of the last dose of all other drugs. The following laboratory measurements were performed at pre-dose: viral load, CD4 cell count, serum AST, ALT, γGT, amylases, alkP, total and direct bilirubine, glucose, cholesterol, HDL, LDL, triglycerides, total magnesium, total calcium, albumin, CK, creatinine, full blood cell count, and pregnancy test.

6.1.3.2. Patients

The study protocol and informed consent form were approved by the local Ethics Committee of the University Hospital Institution. Written informed consent was obtained from all patients. HIV-infected adults were eligible for inclusion if they had been on a stable RAL-containing antiretroviral regimen for at least 3 weeks. Pregnant or breastfeeding women were not eligible.

6.1.3.3. Materials

The solvents used for chromatography and all other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). The phosphate buffered saline (PBS) solution was obtained from Sigma-Aldrich (Schnelldorf, Germany). The RPMI 1640 L-glutamine 25 mM medium and the heat inactivated foetal bovine serum (FBS) used for the isolation of PBMCs from deleukocytation filters for the preparation of matrix (see below) were obtained from Invitrogen (Basel, Switzerland). The Ficoll Separating Solution for the separation of blank PBMCs from other cells was obtained from Biochrom AG (Berlin, Germany).

6.1.3.4. Total plasma and cellular concentrations

RAL, DRV, MVC, ETV and RTV C_{tot} were measured by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS/MS, TSQ Quantum Ion Max, Thermo Fischer Scientific, Waltham, MA, USA) using our validated method.[10] Limit of quantification (LOQ) for C_{tot} in plasma were 7.8 ng/ml for RAL, 10 ng/ml for DRV and ETV, 0.5 ng/ml for MVC and 1 ng/ml for RTV, which correspond to the lowest levels that could be confidently measured with a bias and CV% below $\pm$ 20%. Cell isolation from patient whole blood was performed in Class II biohazard hood, using gloves and long sleeves, according to our previously published method.[1,2] In brief, PBMCs were isolated by density gradient centrifugation in an 8-ml Vacutainer™ CPT tube. After 3 successive washings of cells at +4°C with PBS to eliminate any residual plasma absorbed onto the cell surface, the total PBMCs contained in the pellet were counted in an aliquot using a Coulter instrument (Cell-dyn® 3500R, Abbott AG, Baar, Switzerland). The washed cell pellets were stored at –20°C until drug extraction (MeOH/$H_2$0 50:50). All CPT tubes were processed within five minutes after blood withdrawal, and the total time between blood sampling and washed cell freezing was 2 hours. All sample processing was carried out under ice-cold conditions to prevent drug loss. C_{cell} were determined using an adaptation of our LC-MS/MS method[10] according to the methodology previously described by our group.[1,2] Briefly, calibration samples for cellular determination were prepared using matrix-matched samples containing white blood cells (10^7 cells) isolated from deleukocytation filters obtained from the Hospital Transfusion Unit. On the day of the analysis, white blood cells were spiked with antiretroviral drugs solutions containing internal standard darunavir-d_9 (DRV-d_9) in MeOH/H_2O 50:50 to obtain calibration ranges from 0.025-80 ng/ml for each drug. A 200-µl volume of extracting solution containing DRV-d_9 in MeOH/H_2O 50:50 was added to isolated patient PBMCs pellet. The PBMCs lysates were vortexed, sonicated for 30 minutes for cell lysis and extracted during 30 minutes onto a planar vortexing-vibrating mixer. The extracts were then centrifuged at 14'000 rpm (20'000 g) for 10 minutes at 20°C. A 200-µl volume of supernatant was introduced into a microvial and a 20-µl volume was injected into the HPLC coupled to tandem Mass Spectrometry (HPLC-MS/MS) for drug quantification. C_{cell} were expressed in ng/ml, according to PBMCs cell counts, and assuming a 0.4 pL cell volume.[13]

6.1.3.5. Statistical analysis

The values of PK parameters of RAL, DRV, ETV, MVC and RTV were calculated by non-compartmental pharmacokinetic methods. The maximum concentration observed in plasma (C_{max}), the time to the maximum concentration (T_{max}), the minimum concentration observed in plasma (C_{min}), and the trough (pre-dose) concentration (C_{trough}) were read from the plasma concentration-time curves. The area under the concentration-time curve ($AUC_{0\text{-}12h}$) was calculated over one dosing interval using the linear trapezoid method for C_{tot} and C_{cell} data for each patient. The apparent plasma clearance (CL/F, where F is the bioavailability) was calculated by dividing the administered dose by AUC. Additional PK parameters were also computed: apparent volume of distribution ($Vd/F = CL/F/\lambda_z$) and half-life ($t_{1/2} = 0.693/\lambda_z$), where λ_z is the slope of the terminal elimination phase of the log C_{tot}-time curve.

Geometrical means and coefficients of variations were calculated from the average and standard deviation of log-values. Cellular accumulation was expressed as C_{cell}/C_{tot} ratio. Changes in cellular accumulation over the dosing interval were investigated using ratio values calculated on different times. The correlation between C_{tot} and C_{cell} levels was assessed by longitudinal mixed effects analysis.

6.1.4. Results

6.1.4.1. Patients

Ten patients were enrolled, all of whom had been on stable RAL-containing regimens (400 mg twice daily) for at least 3 weeks. Seven patients were male (70%) and all were Caucasian. Median age was 51 years (range 41 to 67 years), median CD4 cell count was 368 cells/mm3 (range 239 to 839 cells/mm3). Most (9/10) patients had an undetectable viral load (<40 copies/ml) while in one patient the viral load was 68 copies/ml. Co-administered ARVs included DRV/RTV 600/100 mg twice daily (n=6), ETV 200 mg twice daily (n=4), MVC 150 mg twice daily (n=2), EFV 600 mg once daily (n=1), atazanavir 400 mg once daily (n=1), plus emtricitabine/tenofovir (n=5), lamivudine (n=4) or abacavir (n=1).

6.1.4.2. Pharmacokinetic characteristics

RAL, DRV, ETV, MVC and RTV PK parameters determined from observed C_{tot} and C_{cell} values are summarized in Table 1, and the geometric mean (GM) plasma and cellular concentrations versus time profiles are shown in Figure 1. Both concentrations fluctuate in parallel. Coefficients of variation (CV) of PK parameters were important for RAL, and

rather large for other drugs, consistent with the known inter-subject PK variability of ARVs.

Table 1: Total and cellular pharmacokinetic parameters for raltegravir, darunavir, etravirine, maraviroc and ritonavir.

	Raltegravir (n=10)	**Darunavir** (n=6)	**Etravirine** (n=4)	**Maraviroc** (n=2)	**Ritonavir** (n=6)
Total plasma parameters					
AUC (ng*h/ml)	4171	56096	6225	3038	6146
range	825 - 14708	36853 - 94635	3136 - 14120	2276 - 4054	2220 - 9355
CV	160%	48%	86%	50%	70%
C_{max} (ng/ml)	1068	7524	716	408	849
range	172 - 3753	4608 - 11721	488 - 1327	312 - 534	324 - 1378
CV	166%	48%	54%	46%	75%
T_{max} (h)	2.0	2.5	3.6	1.7	3.8
range	1.0 - 6.1	1.0 - 3.0	3.0 - 6.1	1.0 - 3.0	3.0 - 6.1
CV	114%	54%	42%	119%	43%
C_{min} (ng/ml)	51.1	3339	453	128	324
range	8.3 - 345	1580 - 5592	296 - 888	124 - 132	201 - 642
CV	228%	63%	61%	4%	56%
C_{trough} (ng/ml)	40.1	2693	484	128	309
range	8.3 - 345	1580 - 3991	296 - 888	124 - 132	253 - 486
CV	290%	58%	75%	4%	36%
CL/F (L/h)	96	11	32	49.4	16.3
range	27 - 485	6 - 16	14 - 64	37 - 66	10 - 45
CV	160%	48%	86%	50%	70%
$T_{1/2}$ (h)	2.4	7.5	11.5	6.6	5.0
range	1.5 - 3.5	5.5 - 11.0	7.2 - 18.9	4.7 - 9.1	4.0 - 6.7
CV	27%	37%	60%	58%	24%
Vd/F (L)	325	115	533	468	95
range	74 - 1717	52 - 203	315 - 940	253 - 864	71 - 135
CV	191%	79%	73%	138%	31%
Cellular parameters					
AUC (ng*h/ml)	165	7642	9629	8310	11216
range	14 - 906	2429 - 16998	5168 - 17310	6463 - 10685	6922 - 20143
CV	343%	137%	67%	43%	48%
C_{max} (ng/ml)	27.5	1373	1159	1012	1273
range	2.1 - 205	401 - 3704	476 - 3678	782 - 1311	653 - 3480
CV	358%	136%	143%	44%	81%
T_{max} (h)	3.3	4.5	5.5	3.0	3.0
range	3.0 - 8.1	3.0 - 12.0	3.0 - 12.0	-	3.0 - 3.1
CV	37%	86%	100%	1%	1%
C_{min} (ng/ml)	2.9	206	450	419	692
range	0.6 - 26.9	58 - 781	314 - 741	339 - 519	326 - 1491
CV	224%	175%	50%	35%	93%
C_{trough} (ng/ml)	2.7	257	526	419	472
range	0.6 - 26.9	58 - 619	314 - 1398	339 - 519	326 - 739
CV	286%	206%	133%	35%	44%
Accumulation ratios					
C_{cell}/C_{tot} ratio	0.053	0.09	1.29	3.03	1.80
range	0.0067 - 0.37	0.0014 - 0.56	0.60 - 9.90	2.45 - 3.93	0.48 - 7.91
CV	132%	180%	107%	18%	77%
AUC_{cell}/AUC_{tot} ratio	0.039	0.14	1.55	2.74	1.82
range	0.007 - 0.177	0.07 - 0.38	0.82 - 5.52	2.6 - 2.8	0.85 - 5.34
CV	130%	99%	141%	5%	85%

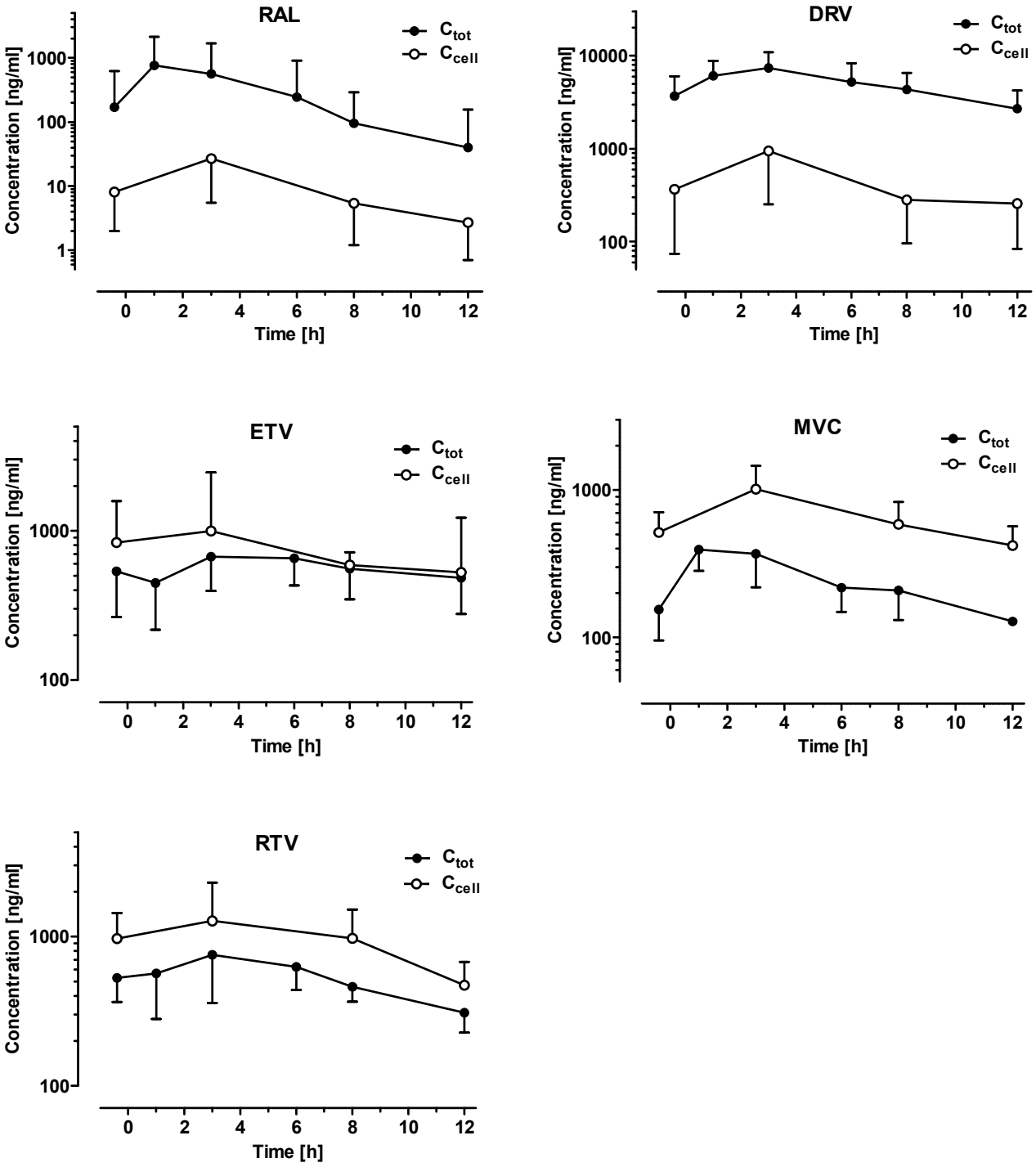

Figure 1: Total and cellular geometric mean concentrations. RAL, raltegravir; DRV, darunavir; ETV, etravirine; MVC, maraviroc; RTV, ritonavir.
Solid and open circles represent total and cellular geometric mean concentrations (geometric SD), respectively.

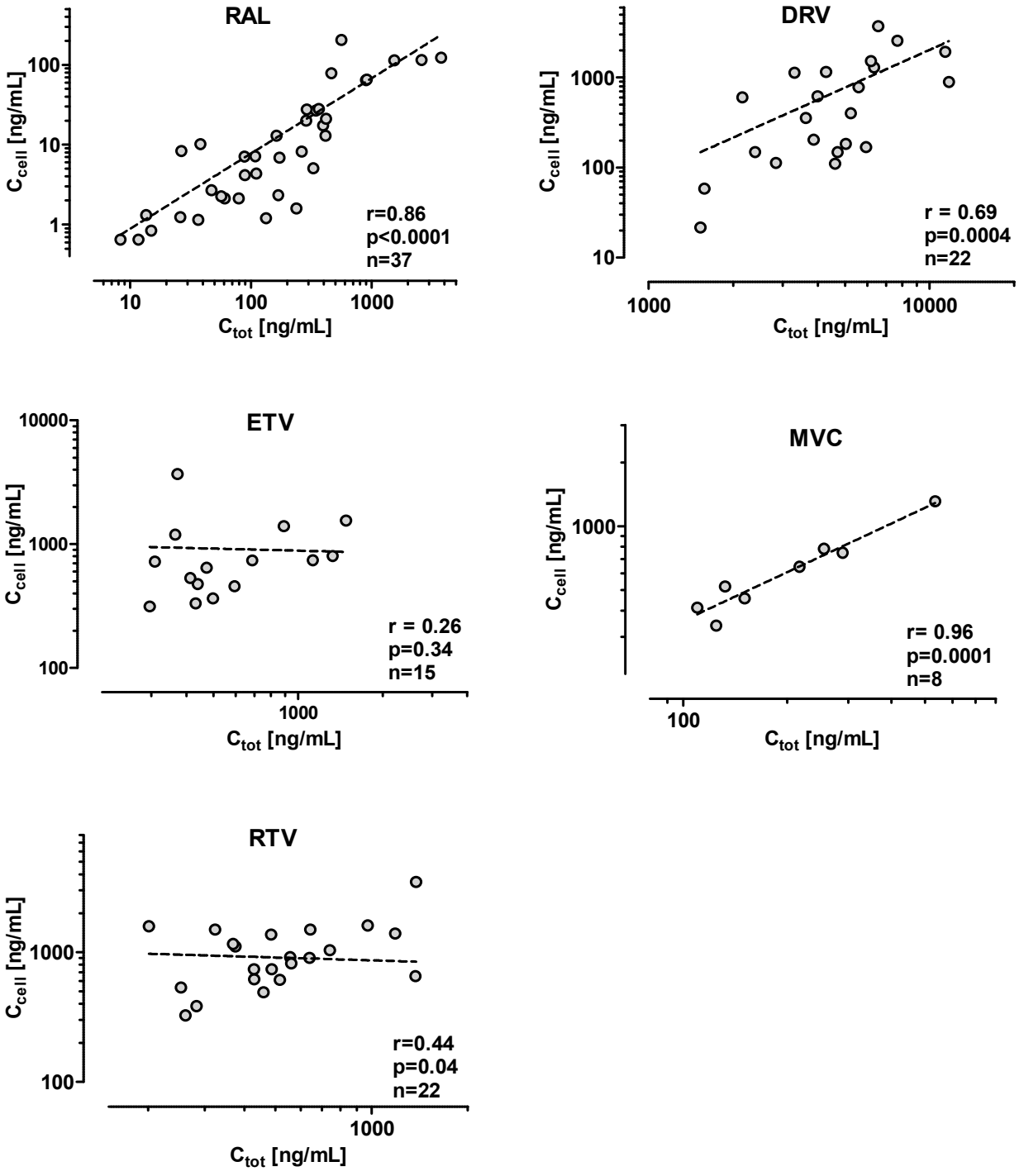

Figure 2: Correlation of C_{cell} versus C_{tot} for raltegravir (RAL), darunavir (DRV), etravirine (ETV), maraviroc (MVC) and ritonavir (RTV).
C_{cell}, cellular concentration; C_{tot}, total plasma concentration; n, number of data; p, P value; r, coefficient of correlation.

6.1.4.3. Correlation between cellular and plasma concentrations

Figure 2 shows the Log-Log linear correlations between C_{tot} and C_{cell} for each drug. Good correlations were obtained for RAL (r=0.86) and MVC (r=0.96) with a slope of the C_{cell}/C_{tot} plot of 0.94 and 0.78, respectively. Correlations were moderate for DRV (r=0.69) and RTV (r=0.44), and poor for ETV (r=0.26) with a corresponding slope of 1.69, 0.45 and 0.34, respectively.

6.1.4.4. Cellular accumulation

RAL C_{cell} (GM) corresponds to 5.3 % of C_{tot} measured simultaneously, with a large inter-patient variability (range 1.3 - 19.6 %). Figure 3 shows the C_{cell}/C_{tot} ratios measured for RAL for each patient at different times after dose administration. C_{cell}/C_{tot} ratios remained stable for each patient without a significant time-related trend over the dosing interval (CV intra = 83%), highlighting the distinct cellular accumulation of RAL in each patient. Conversely inter-individual variability reached 96%, explaining 62% of the total variability. RAL C_{cell}/C_{tot} ratios were not influenced by the presence of other ARVs: 0.044 without vs. 0.059 with comedication with DRV/RTV (p=0.57), 0.051 without vs. 0.055 with comedication with ETV (p=0.89), and 0.050 without vs. 0.065 with comedication with MVC (p=0.66).

AUC_{cell} corresponds to 3.9% (range: 0.7-17.7%) 14% (7-38%), 155% (82-552%), 274% (260-280%) and 182% (85-534%) of AUC_{tot} for RAL, DRV, ETV, MVC and RTV, respectively (Table 1).

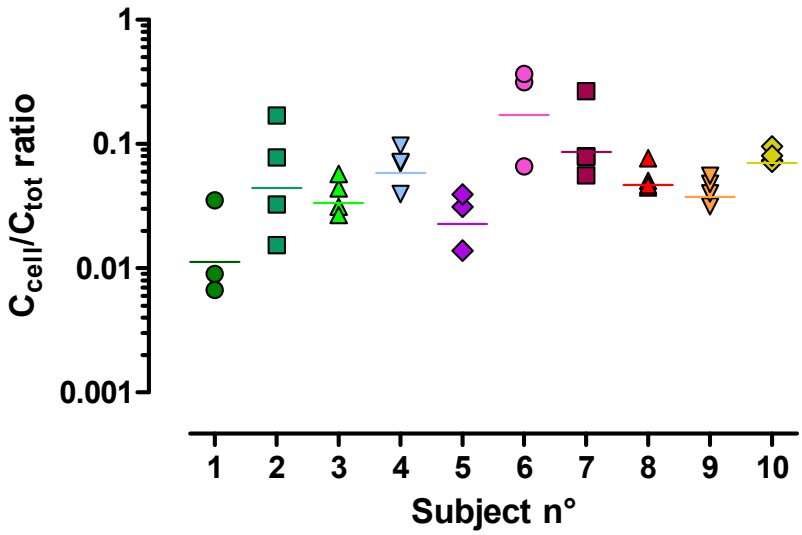

Figure 3: Individual raltegravir Ccell/Ctot ratios measured in the 10 patients at different times after dose intake.

6.1.5. Discussion

Intracellular concentrations of ARVs are most likely a result of passive transport and active uptake and efflux from cells. To date, there has been limited information on the extent of RAL C_{cell}, the impact of co-medications, and whether RAL C_{tot} predicts C_{cell}. The first study published in that field reported no measurable concentrations of RAL in cells (that is, below the limit of quantification of 1 ng/ml of their assay)[14], an unlikely finding for a drug expected to act intracellularly. Conversely, a C_{cell}/C_{tot} ratio for RAL of about 10%, also with a large variability, was recently published in a small group of patients (n=5), using a once a day RAL regimen.[15] In the present study, we confirmed the results from Moltò *et al*.[15] in a patient group of double the size. Further, we expanded our study by examining the importance of inter-individual variability in cellular penetration of RAL and other new ARVs.

We observed that plasma measurements are adequate predictors (r=0.86) for the cellular levels of RAL, without noticeable influence of co-administered ARVs. Drug interaction studies with DRV[16], ETV[17] and MVC[18] have shown a modest, not clinically significant, decrease of about 30% in RAL plasma exposure. This should not affect cell/plasma ratio values because the slope close to unity for RAL indicates that variations in plasma concentrations tend to translate into similar changes in cellular levels. For DRV, the slope was 1.39 and the correlation was less precise (r=0.63, n=22). ETV C_{tot} only moderately reflects the highly variable cellular levels, suggesting that alternative factors (uptake transporters or efflux transporters other than MDR1, ETV being not a P-glycoprotein substrate)[19] may modulate ETV cellular accumulation. As maraviroc is bound into trans-membrane helices of its target CCR5,[20] the membrane-bound drug is analyzed during measurement in PBMCs lysates We found an excellent correlation (r=0.96) between plasma and cellular levels of MVC, although the number of MVC data in our study were limited.

The GM cellular accumulation ratios obtained for DRV and ETV were 0.09 and 1.29, respectively, using concentrations ratios, and are therefore not in agreement with C_{cell}/C_{tot} values of 1.32 and 12.9 previously published for DRV and ETV, respectively.[14] In the latter study by Ter Heine *et al*, however, the plasma used for the determination of total drug concentrations were collected directly from Vacutainer® Cell Preparation Tubes (CPT) tubes. This practice is questionable as Vacutainer® CPT tubes contain liquid components that dilute the plasma phase[21] which in turn will result in lower total plasma concentrations, and hence spuriously high C_{cell}/C_{tot} values. This issue has been verified in our laboratory in a separate set of patients' analyses: RAL total plasma concentrations

(as well as other ARVs) measured in plasma collected from Vacutainer® CPT tubes were 20-30% lower when those determined in parallel directly in EDTA tubes (data not shown). In fact, total plasma levels (and C_{max} values) reported in the Ter Heine *et al.* study[14] were low overall, and were consistently below those previously published for RAL,[1,3,9] DRV,[22-25] ETV[19,24,26] and RTV[25,27]. Besides, a number of unlikely results (no measurable RAL cellular concentration (< 1 ng/ml), more than 15'000 ng/ml of DRV and ETV found in cells) cast some doubt on the reliability of the results of their study. We are confident that our measurements are accurate, since accumulation ratio values determined in our study in parallel to RTV in the same cell pellets (ca 1.80, range 0.48-7.91) are in good agreement with known values previously published for this drug.[2,28,29]

Our study shows that RAL cell penetration is generally low (ca 5% of plasma levels), and that each patient exhibited a distinct cellular accumulation for RAL with C_{cell}/C_{tot} ratios ranging from 0.013 to 0.196, a 15-fold difference. This variability cannot be accounted for by a limitation of analytical performance (precision of 9.3% at the lowest calibrator of 0.025 ng/ml of RAL in PBMCs lysates), nor by other confounding factors (notably cell isolation procedures) likely to affect measurements: DRV, RTV, and ETV, simultaneously determined in the same cell pellets had cellular accumulation ratios variability spanning by only 4- (n= 6 patients), 5- (n=6) and 3.7- (n=4) fold respectively. The 15-fold inter-individual variability in RAL C_{cell}/C_{tot} ratios suggests therefore that alternative factors (transporters or intracellular metabolism) modulate cellular levels of RAL.

So far, because of the small patient numbers exhibiting virological failure in clinical trials on RAL, PK/PD studies have had limited success in finding a clear relationship between clinical response and RAL exposure determined in plasma.[9] Among the few patients who exhibited incomplete viral suppression on an integrase inhibitor-based regimen, most had no genotypic or phenotypic resistance to integrase inhibitor during early virological failure. Resistance emerged only in patients who remained on an integrase inhibitor despite detectable viremia.[30] In those failing patients, RAL concentrations in plasma were either not measured,[30] or, in another study, were found not to be predictive of virological failure, even though most failure cases occurred in patient with low RAL plasma levels.[31] In our study, although limited to virologically-controlled HIV patients (9 out of 10 patients), cellular penetration of RAL was found to be low and characterized by a significant inter-patient variability. RAL cellular accumulation in the single patient for whom VL was not fully suppressed (68 copies/ml, patient 8) did not differ from that in the other patients studied. It is not known at present whether in failing patients a diminished RAL exposure at the expected site of antiviral action may have direct

implications regarding incomplete viral suppression and long-term treatment effectiveness. However, chronic, suboptimal cellular exposure to RAL may in theory permit continuing viral evolution and the progressive emergence of RAL resistance.[30] This is certainly a relevant issue given the almost 10-fold variability in EC_{95} (i.e. 2.7-22.2 ng/ml; 6-50 nM) found in vitro for RAL with clinical isolates from HIV-1 patients' PBMCs.[32]

Recent in vitro studies have shown that the binding of RAL to the preintegration complex (PIC) is essentially irreversible, because the "off rate" (the rate at which RAL dissociates from PIC) is longer than the half-life of the complex.[33] Once binding to the preintegration complex occurs, removing the remaining RAL from culture does not diminish efficacy in vitro. It has been claimed therefore that RAL concentrations measured in patient plasma may be likely irrelevant as long as all intracellular preintegration complexes are bound. However, in studies on raltegravir effects on viral dynamics in patients,[7] the unexpected second phase HIV decay by an integrase inhibitor – not supposed to influence viral production from infected long-lived cells – was explained by RAL effect on both long-lived infected cells and latently infected cells with un-integrated virus.[7] Since total HIV DNA exceed integrated HIV DNA in resting CD4 T cells by 100-fold,[7] it is therefore critical that RAL level in cells remains sufficiently therapeutic to effectively block the new productive infection upon activation of long-lived unintegrated HIV DNA.

Thus, our study suggests that further investigations on RAL cellular disposition are needed in the poorly defined subset of patients whom RAL fails to fully suppress viral replication down to below 40 copies/ml, to investigate the relationships between RAL cellular accumulation in cells, and to study the possible constraints that may restrict the RAL availability to its cellular target and its impact on the levels of both integrated an unintegrated HIV DNA in resting CD4 cells[7]. More generally, because of the original action of RAL, further investigations not only into cellular but also tissue distribution of RAL in various body compartments are warranted, especially in the context of the recent trials of RAL intensification to reduce low-level HIV replication in plasma[34] and gut[35]. So far, these attempts have been of limited success suggesting that residual viraemia is primarily due to HIV release from stable reservoirs (latently infected resting CD4 memory cells and other long-lived cells), but may possibly also arise from some cellular or tissue compartments (i.e. ileum[35]) with ongoing low level replication, for which RAL would have limited – and variable – penetration.

The present study is among the first to provide data on cellular levels of RAL and MVC, but may have some limitations: firstly, the limitation of the pilot study size, secondly the measurements in total PBMCs may only grossly reflect drug penetration in specific target cell populations, such as CD4 T lymphocytes where HIV replicates. Third, the cell volume used for C_{cell} calculation in our study (0.4 pL) may be somewhat overestimated, as recently reported by Simiele *et al.*, who found a lower and more variable volume for PBMCs comprised between 0.23 and 0.34 pL.[36] C_{cell} values reported in our study are therefore conservative: if these recent data on cell volume are confirmed, higher C_{cell} values would be expected. Finally, RAL may be variably embedded in membrane lipid bilayers, complexed to cytoplasmic proteins or sequestrated through intracellular protein binding, as for HIV protease inhibitors,[36] so that only a small fraction of the measured cellular concentrations may remain available to exert the antiviral action. Nevertheless, this *cell-associated* concentration remains the best marker of viral target exposure available at the cellular level. Obviously, drug accumulation within cells is just one of the multiple factors which influence antiviral activity besides drug characteristics (intrinsic potency, affinity for intracellular components and pharmacological target), overall tissue distribution, virus characteristics (susceptibility and genotype) and host factors (genetic background).

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PART IV

CONCLUSIONS

Chapter 7:
Discussion and Perspectives

7.1. Discussion

A prerequisite for any clinical pharmacokinetic study as well as for an efficient Therapeutic Drug Monitoring (TDM) service is the availability of robust bioanalytical methods allowing the accurate and precise determination of drug levels in patients plasma samples with high sensitivity and selectivity.

Section 2.1. reports the development and validation of an LC-MS/MS method for the quantification of the newly approved antiretroviral (ARV) drugs, RAL, DRV, ETV and MVC. This analytical method is currently used within the frame of our routine TDM service for ARV drugs at the Laboratory of Clinical pharmacology at CHUV. As new ARV drugs will continuously be further marketed to widen up antiviral treatment options and improve patient's clinical care, the development of validated analytical method for the monitoring of plasma concentrations will continue to play an essential role for the assessment of correlations between plasma exposure and both efficacy and toxicity.

This LC-MS/MS method was also adapted for the measurement of concentrations of RAL, DRV, ETV and MVC in PBMCs cells isolated from patient's blood, which enables us to describe for the first time the cellular pharmacokinetics of the HIV integrase inhibitor raltegravir, as well as of the CCR5 antagonist maraviroc and the new PI DRV and NNRTI ETV (§ 6.1.). Total plasma RAL concentrations appeared to be well correlated to cellular concentrations (r=0.86), with patients exhibiting low (*ca* 5%) albeit distinct cellular RAL accumulation ratios ranging from 0.013 to 0.196 (a 15-fold difference), suggesting therefore that alternative factors (transporters or intracellular metabolism) modulate cellular levels of RAL. This significant inter-patient's variability observed for RAL cell accumulation should prompt further investigations in patients failing in an integrase inhibitor-based regimen.

Within the frame of another clinical study, a new optimised ultrafiltration procedure followed by LC-tandem MS has been developed for the determination in patients of free plasma concentrations of the major PIs and NNRTIs, as reported in Section 2.2. The LC-tandem MS technique has been used successfully applied for the monitoring of not only

total but also free – pharmacologically active – plasma concentrations of LPV, ATV and NVP in HIV-infected pregnant women followed during the course of pregnancy. We have demonstrated that total as well as free plasma exposure of LPV, ATV and NVP were not influenced to a clinically significant extent by pregnancy. Systematic dosage adjustment for this drug is therefore not required in HIV-infected pregnant women.

Our LC-MS/MS assay for antiretroviral drugs has been also applied during a prospective study evaluating the effectiveness of TDM to guide EFV dose reduction in patients having EFV concentrations above the recommended therapeutic range. TDM-guided dosage adjustment of EFV using a simplified algorithm based on a Bayesian approach was a successful and safe strategy, without negative impact on clinical outcomes. Of note, EFV dosage reduction could have been predicted with nearly similar precision by genotyping patients for the relevant polymorphisms of EFV metabolising enzymes CYP2B6, 2A6 and 3A4/5. Finally, even if this was not a primary endpoint of the study, downward adjustment of EFV dose based on TDM in patients with plasma concentration above therapeutic target may also have an important impact for reducing treatment costs.

The impact of genetic polymorphisms of CYP2B6, 2A6 and 3A4/5 on the PK of EFV and its metabolites was also studied by a comprehensive approach using PG-based popPK and integrating the additional information of patients metabolite profiling. A population PK model was built integrating both genetic and demographic covariates which allowed us to identify most part of the remaining variability in EFV exposure that remained unexplained by genetic variations in *CYP2B6*. Indeed, the role of the accessory pathway CYP2A6 appeared to be critical in limiting drug accumulation in individuals with reduced CYP2B6 activity. Functional genetic variations in main (CYP2B6) and accessory (CYP2A6, 3A4/5) metabolic pathways of EFV have therefore an impact on EFV disposition, and may lead to extreme EFV plasma exposures. Dosage adjustment guided by TDM is thus required in those patients, according to the pharmacogenetic polymorphism.

Throughout this work, we aimed at demonstrating that a better understanding of pharmacokinetic and pharmacogenetic factors influencing ARV drugs exposures will help us to provide a rational basis for optimising the ARV therapy of HIV-infected patients. The adequacy between drug dose, plasma exposure and clinical outcome has been evaluated in the two main studies of the present thesis. In patients with excessive EFV

plasma levels, treatment optimisation can be achieved by TDM-adjusted drug dose reduction according to plasma levels, or using an EFV dosage reduction scheme based on patient pharmacogenetic background. Alternately in another situation, total and free plasma levels of ARV drugs appear not to be decreased to a clinically significant extent in HIV-positive pregnant women, implying therefore that an unchanged antiretroviral drug dosage during pregnancy is probably the best treatment recommendation at present. Finally, our finding on the very low and distinct disposition of the integrase inhibitor RAL in the compartment where HIV replicates should be the basis of further cellular studies in the few patients failing on a RAL-based regimen, for reasons largely unknown at present.

These are all relevant examples where individualised therapy certainly provides a significant benefit for the patients in terms of tolerability to ARV treatments, improving thereby patient adherence to antiretroviral therapy, which in turn influence treatment long-term effectiveness.

Being able to propose the optimal ARV treatment tailored to each individual patient not only positively influence individual patient's care but has also a beneficial impact on health care system and the whole society overall. To reduce the likelihood of vertical transmission, HIV-infected pregnant women have to be virologically suppressed at delivery: this can only be obtained in case of strict adherence to treatment, which is obviously highly dependant on how well HIV-infected pregnant women tolerate their antiretroviral drugs regimen. In another context, the treatment optimisation through the reduction of EFV doses in patients with excessive – unnecessary – drug exposure not only improve long term tolerability but have indirect beneficial consequence in terms of treatment costs, and drugs resources utilisation.

In conclusion, the overall objective of this work was the optimisation of antiretroviral therapy by treatment individualisation. The overall benefit of such an approach appears to be increasingly recognized. However, it will certainly necessary to pursue this effort for decades until, one day, we will overcome HIV.

7.2. Perspectives

During this work, a large amount of information has been accumulated in the context of antiretroviral therapy optimisation, and only a part of these data has been presented here within the frame of my PhD thesis.

In fact, analyses of available data are ongoing but remain yet to be completed, notably a comprehensive population pharmacokinetics of lopinavir in HIV-infected pregnant women using NONMEM.

Another study not presented in this thesis, but for which we have been also implicated aimed at optimising raltegravir plasma exposure via a potentially beneficial ARV drug interaction with ATV known to inhibit UDP-glucuronosyltransferase 1A1 (UGT1A1), the main metabolising enzyme of RAL. A pharmacokinetic study on the RAL - ATV association in healthy volunteers has been initiated under the auspices of Médecins sans Frontières with the collaboration of the University of South California at Los Angeles (USCLA). The LC-MS/MS quantification of RAL, RAL-glucuronide and ATV in clinical samples has been carried out in our Laboratory. Pharmacokinetic analysis is currently performed. It is planned to compare the results obtained with the pharmacokinetic approaches used at USCLA and in Lausanne.

Finally, in the context of our collaboration with the Institute of Microbiology at CHUV and with the Geneva Clinical Pharmacy Unit attached to the Division of Clinical Pharmacology at CHUV, extensive pharmacokinetics-pharmacogenetics studies for ETV and RAL have been initiated. More specifically, it is anticipated that we will actively participate to the development of RAL population pharmacokinetics.

APPENDICES

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A LC–tandem MS assay for the simultaneous measurement of new antiretroviral agents: Raltegravir, maraviroc, darunavir, and etravirine

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ABSTRACT

Raltegravir (RAL), maraviroc (MVC), darunavir (DRV), and etravirine (ETV) are new antiretroviral agents with significant potential for drug interactions. This work describes a sensitive and accurate liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the determination of plasma drug levels. Single-step extraction of RAL, MVC, DRV, ETV and RTV from plasma (100 μl) is performed by protein precipitation using 600 μl of acetonitrile, after the addition of 100 μl darunavir-d_9 (DRV-d_9) at 1000 ng/ml in MeOH/H_2O 50/50 as internal standard (I.S.). The mixture is vortexed, sonicated for 10 min, vortex-mixed again and centrifuged. An aliquot of supernatant (150 μl) is diluted 1:1 with a mixture of 20 mM ammonium acetate/MeOH 40/60 and 10 μl is injected onto a 2.1 × 50 mm Waters Atlantis™-dC18 3 μm analytical column. Chromatographic separations are performed using a gradient program with 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytes quantification is performed by electrospray ionisation-triple quadrupole mass spectrometry using the selected reaction monitoring detection in the positive mode. The method has been validated over the clinically relevant concentrations ranging from 12.5 to 5000 ng/ml, 2.5 to 1000 ng/ml, 25 to 10,000 ng/ml, 10 to 4000 ng/ml, and 5 to 2000 ng/ml for RAL, MRV, DRV, ETV and RTV, respectively. The extraction recovery for all antiretroviral drugs is always above 91%. The method is precise, with mean inter-day CV% within 5.1–9.8%, and accurate (range of inter-day deviation from nominal values −3.3 to +5.1%). In addition our method enables the simultaneous assessment of raltegravir–glucuronide. This is the first analytical method allowing the simultaneous assay of antiretroviral agents targeted to four different steps of HIV replication. The proposed method is suitable for the Therapeutic Drug Monitoring Service of these new regimen combinations administered as salvage therapy to patients having experienced treatment failure, and for whom exposure, tolerance and adherence assessments are critical.

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1. Introduction

Therapeutic interventions in HIV infection have been up to now mainly directed towards two viral enzymes, reverse transcriptase and protease. Despite the clinical efficacy of multiple drug combination treatments generally observed with current HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), resistant HIV strains appearance continuously necessitates alternative antiretroviral regimens. Development of new agents has expanded the number of available molecules in the currently available classes as well as in novel classes. The new agents are of particular interest for treatment-experienced patients with multidrug-resistant HIV for whom therapeutic options are limited.

Raltegravir (RAL; Isentress®) is the first member of the long-anticipated class of HIV integrase inhibitors that has reached the final stage of clinical development. Raltegravir has antiviral activity in both naïve [1,2] and antiretroviral-experienced patients [3–5] with mostly favourable safety profile so far. Alternately, new therapeutic approaches directed to host, not viral, targets are also emerging: maraviroc (MVC; Celsentri®, Selzentry®) is a chemokine CCR5 co-receptor antagonist [6–9] that is used at present, in

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experienced, R5-tropic HIV-infected patients for whom previous antiretroviral regimens have failed.

Darunavir (DRV, TMC114; Prezista®) is a PI of the latest generation characterized by a good activity against multi-drug resistant viral isolates and is used in association with low-dose ritonavir, in experienced patients with limited treatment options [10,11].

Finally, etravirine (ETV, TMC125; Intelence®) is a novel chemical class of NNRTIs with potent *in vitro* activity against both wild-type and some resistant HIV mutants (notably the K103N mutants) to currently approved NNRTIs.

Various combination regimens with darunavir, etravirine and/or raltegravir are currently being studied: the DUET studies (darunavir and etravirine) [12–15], and more recently, the TRIO study (darunavir plus etravirine and raltegravir) with promising results [16].

Among these four drug classes, Therapeutic Drug Monitoring (TDM) of NNRTIs and PIs yields some clinical benefit for the management of antiretroviral treatment [17]. Whether TDM is also beneficial for the new classes of integrase inhibitors and CCR5 antagonists remains to be established, but can be anticipated considering their metabolic pathways and the drug interaction potential of complex multiple agent-based regimen. The HIV integrase inhibitor raltegravir is primarily metabolized by uridine-5′-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) [18], an enzyme that is characterized by genetic polymorphism and that can also be inhibited by the PI atazanavir [19]. The CCR5 antagonist maraviroc is a P-glycoprotein substrate and is extensively metabolized by the CYP3A isoform pathways. As a consequence, maraviroc plasma Area-Under-the-Curves (AUCs) are enhanced in the presence of potent inhibitors (ritonavir) and decreased in the presence of CYP3A inducers such as the NNRTIs efavirenz or etravirine, requiring maraviroc dosage adjustment [20]. Finally, the novel PI darunavir, mainly metabolized by CYP3A4 isoform, is used in association with the CYP3A inhibitor RTV as pharmacoenhancer and DRV/r combination is therefore at similar risk of the various drug interactions potential as most regimens with boosted PIs.

All these new agents therefore have the potential for significant reciprocal drug–drug interactions not to mention those with other antiretroviral medications, adding to the challenges of constructing long-term effective combination antiretroviral regimens. To that endeavour careful monitoring of drug exposure of these new drugs seems to be an important component of patients follow-up.

To date, several methods have been published for the assay of raltegravir by HPLC coupled to fluorescence [21] or photodiode array detection, with other antiretroviral drugs [22], or by LC–MS/MS alone [23,24] or together with its glucuronide metabolite [18,25]. Reports have also been published recently describing chromatographic methods for the assay of darunavir by HPLC–UV [22,26] or by LC–MS/MS, together with other antiretroviral drugs [27–30]. Assays for maraviroc have been described, as part of pre-clinical and clinical studies, without however much details on the validation of the analytical method [31–35]. Finally, even though analytical methods have previously been developed for etravirine within the frame of drug interactions studies [14,15,36], only one formal validation of an HPLC method using off-line solid-phase extraction, followed by reversed-phase chromatography and UV photodiode array detection has been reported so far for the measurement of etravirine, along with raltegravir, darunavir and several other antiretroviral agents [22]. This method, however, was unable to achieve a chromatographic separation for darunavir and amprenavir which co-eluted as a single peak. Recently, a LC–tandem MS has been proposed for the determination of etravirine in various biological matrices [37].

Complex salvage therapy regimens given to failing patients often combine raltegravir with boosted darunavir and etravirine, and possibly maraviroc in CCR5-tropic viral infection. Such regimens mandate therefore an analytical method enabling the unambiguous measurement of these various antiretroviral drugs altogether.

In this report, we therefore describe the development and validation of a straightforward analytical method for the simultaneous analysis in plasma of the four most recently licensed new antiretroviral agents raltegravir, maraviroc, darunavir, etravirine (chemical structures in Fig. 1) and ritonavir by liquid chromatography coupled with tandem triple quadrupole mass spectrometry detection. This method is characterized by a very low limit of quantification, below the clinically relevant range of concentrations encountered in patients. Interestingly, our method enables the simultaneous determination of raltegravir–glucuronide, giving some insight into the role of the UGT1A1-mediated metabolism of raltegravir. This method is currently applied in our TDM Service for patients' follow-up and for clinical research projects within the frame of the Swiss HIV Cohort Study.

2. Experimental

2.1. Chemicals and reagents

Raltegravir potassium (RAL) was kindly provided by Merck (Rahway, NJ, USA), maraviroc base (MVC) by Pfizer (New York, USA), darunavir ethanolate (DRV) and etravirine base (ETV) by Tibotec (Mechelen, Belgium) and ritonavir base (RTV) by Abbott (Baar, Switzerland). Darunavir-d_9 (DRV-d_9) purchased from LGC Prochem (Molsheim, France) was used for internal standard (I.S.) for the assay.

Acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH), all LiChrosolv® grade, and 100% formic acid were purchased from Fluka (Buchs, Switzerland). All other chemicals (including ammonium acetate) were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA).

Blank plasma used for the assessment of matrix effect and for the preparation of calibration and control samples were isolated (1850 g, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from blood withdrawn from patients with Vaquez disease.

2.2. Equipment

The high-performance liquid chromatography system involved a Rheos 2200 binary pump (Flux Instruments, Basel, Switzerland) equipped with an online degasser and a temperature-controlled 324 vial autosampler maintained at +10 °C (CTC Analytics AG, Zwingen, Switzerland). Separations were done on a 2.1 mm × 50 mm Waters Atlantis™-dC18 3 μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 25 °C (Croco-Cil, Cluzeau Info Laboratory, Courbevoie, France). The chromatographic system was coupled to a triple-stage quadrupole mass spectrometer (TSQ Quantum) from Thermo Fisher Scientific (Waltham, MA) equipped with an Ion Max electrospray ionisation interface and operated of Xcalibur software package (version 2.0) (ThermoQuest, Thermo Fisher Scientific Inc, Waltham, MA).

2.3. Solutions

2.3.1. Mobile phase solutions

Solution A consisted of 2 mM ammonium acetate in ultrapure water containing 0.1% formic acid (FA) (pH 2.8). Solution B consisted of 0.1% formic acid in MeCN. Solvents were regularly prepared prior to each series of analysis.

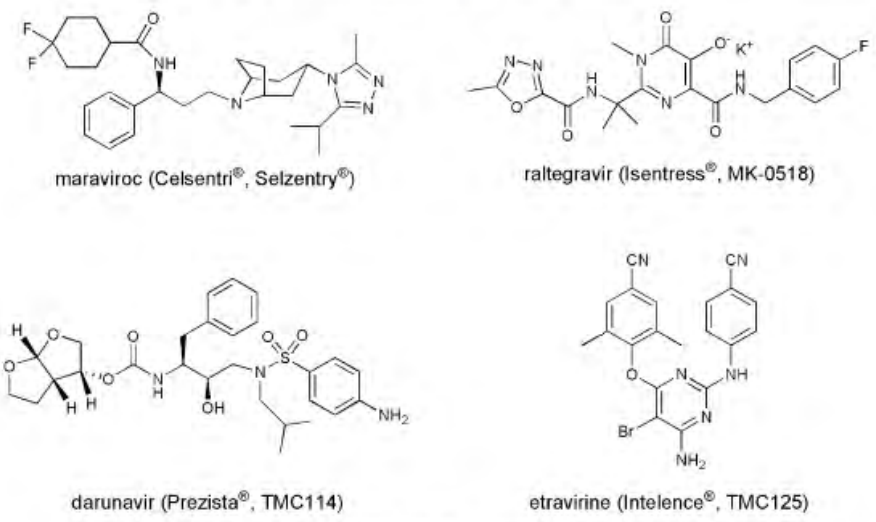

Fig. 1. Chemical structures of new antiretroviral drugs.

2.3.2. Internal standard, calibration standard and quality control (QCs) solutions

A stock solution of the internal standard (I.S.) darunavir-d_9 (DRV-d_9) at 1 mg/ml (in MeOH) was diluted with MeOH/H_2O 50/50 (vol/vol) to obtain a working solution at 1000 ng/ml.

Stock solutions of MVC (0.2 mg/ml in MeOH), DRV and RTV (1 mg/ml in MeOH), ETV (1 mg/ml in EtOH + FA, to pH 4.2) and RAL (2 mg/ml in MeOH/H_2O 50/50) were diluted with MeOH/H_2O 50/50 for the preparation of working solutions at concentrations ranging from 50 to 200,000 ng/ml (depending on the antiretroviral drug). These working solutions were diluted 1:20 with blank plasma to obtain the calibration samples ranging from 2.5 to 10,000 ng/ml and the corresponding three quality control samples (low (L), medium (M) and high (H) QCs) from 25 to 7500 ng/ml (Table 1). All solutions were prepared according to the recommendations on bioanalytical methods validation stating that the total added volume must be ≤10% of the biological sample volume [38,39]. The calibration and QCs samples were stored as 1-ml aliquots at −20 °C prior to analysis.

2.4. LC–MS/MS conditions

The mobile phase was delivered using a stepwise gradient elution program: 2% of B at 0 min, 30% of B at 3.2 min, 100% of B at 10.0 min, with a flow-rate of 0.3 ml/min. The second part of the run includes an intensive rinsing (100% of B at 10.1 min with 0.5 ml/min) for 5 min and a re-equilibration step to the initial solvent composition up to 20 min (at 15.1 min with 0.4 ml/min and at 18.0 min with 0.3 ml/min). The thermostated column heater was set at 25 °C and the autosampler was maintained at 10 °C. The injection volume was 10 μl.

The mass spectrometer was operated with the electrospray ionisation source Ion Max in the positive mode. Samples were analysed via selected reaction monitoring (SRM) detection mode, employing the transition of the $[M+H]^+$ precursor ions to product ions. The selected *m/z* transitions and the collision energy (CE) for each analyte are reported in the Table 2.

The determination of optimal potential settings and MS/MS transitions were chosen by direct infusion into the MS/MS detector of a MeOH/H_2O 50/50 solution of all drugs at a concentration of 1 μg/ml. The first (Q1) and third (Q3) quadrupoles were set at 1 amu mass resolution (full-width half-maximum = 0.7), except for ETV, for which the full-width half-maximum was 1.5 (= 2.1 amu mass resolution). Scan time and scan width were 0.04 s and 1.0 *m/z*, respectively, and each chromatographic peak was the result of around 30 scans.

The ionisation conditions were as follows: the capillary temperature was set at 350 °C. The ESI spray voltage was set at 4 kV, the source induced dissociation was set at 10 V. The sheath and auxiliary gas (nitrogen) flow-rate was set at 35 and 10 (arbitrary units), respectively. The tube lens voltages range from 67 to 123 V and the Q2 collision gas (argon) pressure was 1 mTorr.

Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur LC-Quan software package.

2.5. Plasma sample preparation

2.5.1. Selection of the reconstitution solvent

During the initial development of the method, the following solvents were evaluated for the reconstitution of the plasma extract supernatant: 20 mM ammonium acetate/MeOH 50/50, 40/60 and

Table 1
Concentration of working solutions and preparation of calibration and QC samples.

Drug	Stock solution solvent	Stock solution concentration	Working solution concentration (obtained by dilution of stock solution with MeOH/H_2O 50/50)	Calibration range (obtained by dilution of working solution with plasma 1:20)	QCs controls
Darunavir (DRV)	MeOH	1 mg/ml	500–200,000 ng/ml	25–10,000 ng/ml	250; 1250; 7500 ng/ml
Etravirine (ETV)	EtOH	1 mg/ml	200–80,000 ng/ml	10–4000 ng/ml	100; 500; 3000 ng/ml
Maraviroc (MVC)	MeOH	0.2 mg/ml	50–20,000 ng/ml	2.5–1000 ng/ml	25; 125; 750 ng/ml
Raltegravir (RAL)	MeOH/H_2O 50/50	2 mg/ml	250–100,000 ng/ml	12.5–5000 ng/ml	125, 625; 3750 ng/ml
Ritonavir (RTV)	MeOH	1 mg/ml	100–40,000 ng/ml	5–2000 ng/ml	50; 250; 1500 ng/ml

Appendix 2.1.

Table 2
Instrument method for the LC–MS/MS analysis for RAL, MVC, DRV, RTV and ETV with DRV-d_9 as internal standard.

Drug	Parent (*m/z*)	Product (*m/z*)	CE (eV)	Tube lens (V)	Typical RT (min)
Darunavir (DRV)	548.3	392.0	25	75	7.4
Etravirine (ETV)	434.9	303.9	49	111	9.0
Maraviroc (MVC)	514.3	280.0	40	87	5.6
Raltegravir (RAL)	445.1	361.0	27	84	6.7
Ritonavir (RTV)	721.4	296.2	26	123	8.2
Darunavir-d_9 (DRV-d_9)	557.3	401.2	25	67	7.4

CE, collision energy; mean RT, retention time; MS acquisition time (min), 15.25; Q2 collision gas pressure (mTorr), 1.00.

30/70. Among the solvent mixtures tested, the 40/60 provided the best chromatographic behaviour and peaks area intensity overall for RAL, MVC, DRV, RTV, ETV and the I.S. DRV-d_9, and was consequently used thereafter throughout the method.

2.5.2. Extraction procedure

A 100-μl aliquot of plasma sample was mixed with 100 μl of I.S. solution (1000 ng/ml DRV-d_9 in MeOH/H_2O 50/50) and 600 μl of MeCN, vortexed and sonicated for 10 min (Branson Ultrasonics Corporation, Danbury, CT, USA). The mixture was again vortex-mixed and finally centrifuged at +4 °C for 10 min at 20,000 g (14,000 rpm) on a Hettich Benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). A 150-μl aliquot of the supernatant was diluted 1:1 with 20 mM ammonium acetate/MeOH 40/60 into 500 μl glass HPLC microvials and after secure closing with crimp seals finally vortexed. A volume of 10 μl was used for LC–MS/MS analysis.

2.6. Quantification

2.6.1. Calibration curves

Quantitative analysis of the five antiretroviral drugs was performed using the internal standard method. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared with plasma isolated from outdated blood transfusion bags.

A seven-point calibration standard curve was calculated and fitted either by $1/x$ or $1/x^2$ weighted quadratic regression, or quadratic log–log regression, when appropriate, of the peak area ratios (drug peak area/I.S. peak area) versus concentrations. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The calibration for the five antiretroviral drugs was established over the range reported in Table 1, so as to cover the range of clinically relevant concentrations expected in patients. Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of Xcalibur software package (version 2.0) (ThermoQuest, Thermo Fischer Scientific Inc, Waltham, MA).

2.7. Analytical method validation

The method validation procedure was based on the recommendations published on-line by the FDA [38] as well as on the updated recommendations of the Conference Report of the Washington Conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies" [39].

2.7.1. Accuracy and precision

Replicate analysis ($n = 6$) of QC samples at the three concentrations (low (L), medium (M), high (H)) (Table 3) were used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by repeated analysis performed on six different occasions (Table 3). The concentration in each sample was determined using calibration standards prepared on the same day. The precision was calculated as the coefficient of variation (CV %) within a single run (intra-assay) and between different assays (inter-assay), and the accuracy as the percentage of deviation between nominal and measured concentrations.

During the routine analysis of patient samples, control samples at each QC concentration levels were assayed. The analytical series were considered valid and accepted only if the percentage of deviation (bias) between nominal and back-calculated (experimental) concentrations for each calibration level and quality control samples were less than ±15%, and less than ±20% at the limit of quantification (defined as the lowest calibrator).

2.7.2. Limit of quantification and limit of detection

Serial dilutions (1/2, 1/5, 1/10, 1/16, 1/25, 1/50) of the lowest QC sample were analysed in triplicates. The lowest limit of quantification (LLOQ) for each drug analysed was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the documents mentioned above, recommending that the deviation between measured and nominal concentrations should not deviate more than ±20%. Calibration curves were established with calibration standards including either one or the other of dilution samples. The LLOQ concentrations were finally selected as the lowest levels of the calibration curves with a bias and CV% below ± 20%.

A second set of dilutions (1/10, 1/20, 1/40, 1/50, 1/100, 1/200, 1/400) of the lowest QC sample and a blank plasma extract were analysed to determine the limit of detection (LOD), defined as the concentration that produced a signal three times above the noise level of a blank preparation.

2.7.3. Stability of antiretroviral drugs

Stability studies of RAL, MVC, DRV, ETV and RTV included:

a) Stability of plasma spiked with these antiretroviral drugs kept at room temperature (RT) and in the fridge at +4 °C: the concentrations were measured immediately after preparation and after being left at room temperature (RT) and at +4 °C up to 48 h. Antiretroviral drugs concentrations variations were expressed as a percentage of the nominal concentration.
b) Stability of plasma samples after multiple freeze–thaw cycles: QC samples at L, M and H levels of antiretroviral drugs underwent three freeze–thaw cycles. Frozen samples were allowed to thaw at room temperature for 1 h and were subsequently refrozen for 2 h. Antiretroviral drugs concentrations were measured in aliquots from the three consecutive freeze–thaw cycles. The variations of concentrations were expressed as a percentage of the initial concentration measured at the beginning of the stability study.
c) Stability of plasma samples kept frozen at −20 °C: The response of freshly prepared plasma calibration and QC samples was compared to those of frozen calibration and QCs samples stored during 6 weeks at −20 °C.

Table 3
Precision and accuracy of the assay for the five antiretroviral drugs in human plasma with QC samples at low, medium and high concentrations.

		Inter-assay (n = 6)				Intra-assay (n = 6)			
	Nominal conc. (ng/ml)	Concentration found (ng/ml)	SD ±	Precision CV%	Accuracy bias%	Concentration found (ng/ml)	SD ±	Precision CV%	Accuracy bias%
Darunavir	250	253.6	18.2	7.2	1.4	257.6	18.8	7.3	3.0
	1250	1313.5	67.7	5.2	5.1	1261.7	85.2	6.8	0.9
	7500	7400.6	482.2	6.5	−1.3	7715.6	355.2	4.6	2.9
Etravirine	100	103.5	8.3	8.1	3.5	94.1	6.3	6.7	−5.9
	500	483.3	33.2	6.9	−3.3	500.1	27.6	5.5	0.0
	3000	2945.5	225.0	7.6	−1.8	3291.2	108.6	3.3	9.7
Maraviroc	25	26.3	2.4	9.2	5.0	24.5	1.6	6.4	−2.1
	125	122.5	6.3	5.1	−2.0	124.7	9.0	7.2	−0.3
	750	727.6	62.3	8.6	−3.0	804.8	28.1	3.5	7.3
Raltegravir	125	129.3	12.6	9.8	3.5	122.9	8.7	7.1	−1.7
	625	604.7	35.8	5.9	−3.3	617.8	29.3	4.7	−1.2
	3750	3673.8	302.6	8.2	−2.0	3697.2	162.4	4.4	−1.4
Ritonavir	50	51.5	4.2	8.1	2.9	50.5	4.5	8.9	1.0
	250	245.9	17.0	6.9	−1.6	245.8	16.4	6.7	−1.7
	1500	1484.2	143.3	9.7	−1.1	1552.0	136.8	8.8	3.5

2.7.4. Matrix effect and recovery

In the initial step of method validation, the matrix effect was examined qualitatively by the simultaneous post-column infusion of the five antiretroviral drugs and the I.S. into the MS/MS detector during the chromatographic analysis of blank plasma extracts. The standard solution of all analytes at 100 ng/ml was infused at a flow-rate of 10 μl/min during the chromatographic analysis of six blank plasma extracts from six different sources. The chromatographic signals of each selected MS/MS transition were examined to ascertain that no major signal perturbation (drift or shift) of the MS/MS signal was present at the analyte's retention time.

Subsequently, the quantitative determination of the matrix effect, and the determination of its variability were also assessed. Three series of QC samples at L, M and H concentration in duplicates were prepared as followed:

(A) Pure standard solutions of antiretroviral drugs, subjected to the same extraction/buffer dilution as for plasma, directly injected onto column;
(B) Blank plasma extract samples from six different sources, spiked with antiretroviral drugs and I.S. *after* extraction;
(C) Plasma samples from six different sources (same as in (B)) spiked with QC standard solutions and I.S. *before* extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effect) was assessed by comparing the absolute peak areas of analytes either solubilised in extraction/buffer medium (A), or added after (B) and before (C) extraction of six different batches of plasma, based on the recommendations proposed by Matuszewski et al. [40,41].

The *extraction yield* of antiretroviral drugs and I.S. was calculated as the absolute peak-area response in processed plasma samples spiked with drugs *before* extraction (C), expressed as the percentage of the response of the same amount of drugs added into blank plasma extracts *after* the extraction procedure (B) (C/B ratio in %). The *matrix effect* was assessed as the ratio of the peak areas of analytes added into blank plasma extracts *after* the extraction procedure (B) to the peak areas of pure analytes solubilised in extraction/buffer medium (A) (B/A ratio in %). The *overall recovery* of antiretroviral drugs and I.S. was calculated as the ratio of absolute peak-area response of antiretroviral drugs either in processed plasma samples spiked with drugs *before* extraction (C) to the peak areas of analytes solubilised in extraction/buffer medium (A) (C/A ratio in %).

2.7.5. Influence of plasma matrix variability on raltegravir–glucuronide/raltegravir ratios values

Raltegravir–glucuronide pure standard was not available to us and our method allows therefore only a relative (rather than absolute) measurements of raltegravir–glucuronide levels in patients, assessed by the raltegravir–glucuronide/raltegravir (RAL–gln/RAL) ratio. Whether the variability in patients' plasma matrix significantly influences the efficiency of raltegravir–glucuronide in-source dissociation, and hence the RAL–gln/RAL ratios values, was verified with the following experiments: plasma from HIV patients on raltegravir with high (>2.5), elevated (>1.0) and low (<0.3) RAL–gln/RAL ratios were diluted 1:2 with blank plasma from six different sources (healthy volunteers, Vaquez patients). The RAL–gln/RAL ratios measured in these patient samples before and after the addition of various plasma matrices were compared. In fact, this comes to study the matrix effect variability at the retention time of RAL–gln, similarly to what is done for the other drugs (see Table 6).

2.7.6. Dilution effect

Some patient samples were found to contain drug concentrations exceeding the high level of the calibration curve (see Table 1). To ascertain whether the dilution of these samples prior to a subsequent analysis could affect the accuracy of the drug determination, a blank plasma sample was spiked with antiretroviral drugs at a concentration exceeding by two-fold the highest calibration level. The sample was thereafter analysed in triplicate after a five-fold dilution to bring the concentration within the calibration range. Dilution was carried out with blank plasma. Calculated and expected concentrations were compared.

3. Results

3.1. Chromatograms

The proposed method enables the simultaneous quantification of the latest generation antiretroviral drugs (RAL, MVC, DRV, ETV) and RTV by liquid chromatography coupled with tandem MS/MS. Typical chromatographic profiles of calibration and QC samples containing RAL, MVC, DRV, ETV and RTV at concentrations corresponding to the lowest calibration level (i.e. corresponding to

Appendix 2.1.

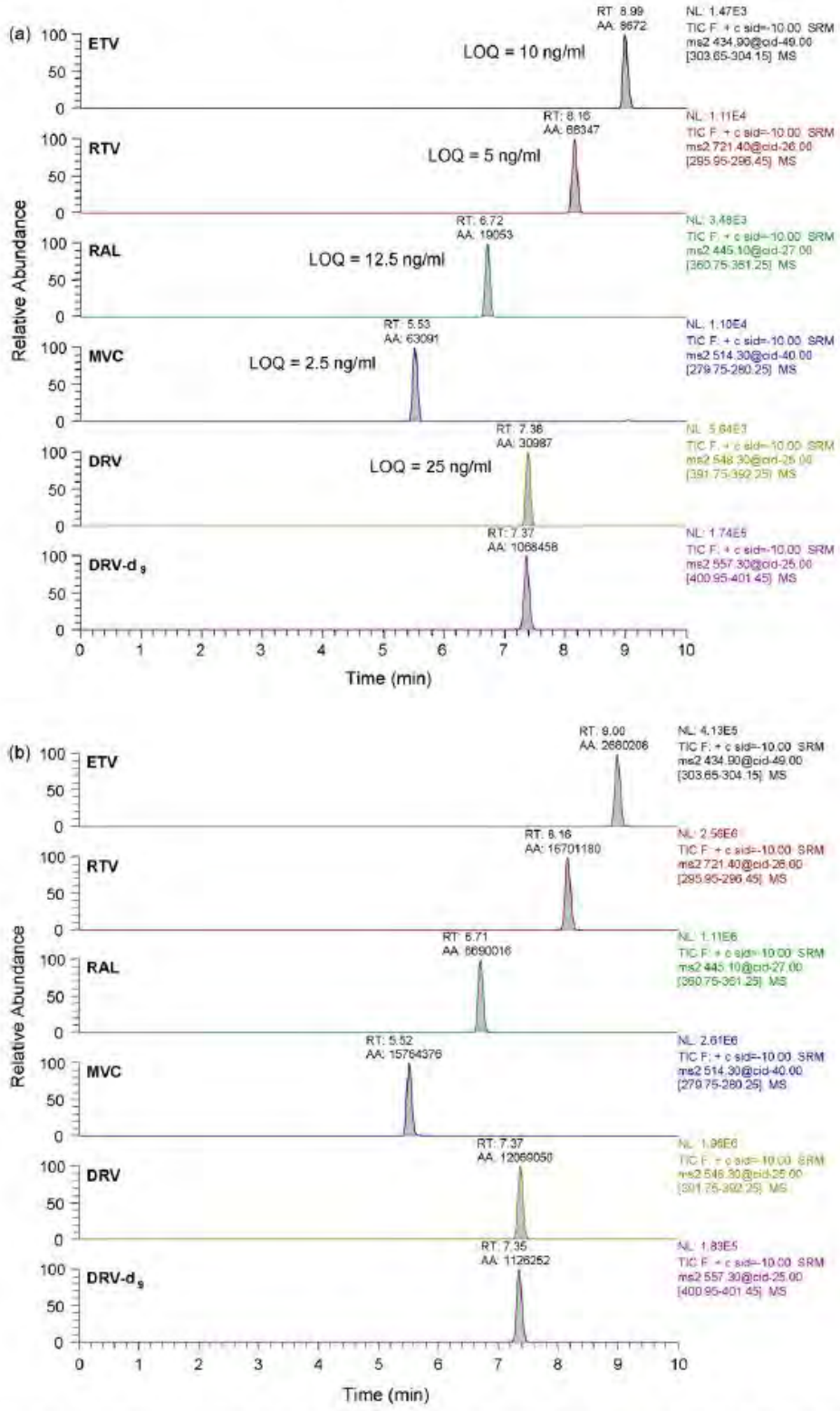

Fig. 2. Chromatograms of (a) the lowest calibration sample (LOQ) and (b) the highest quality control sample containing DRV, ETV, MVC, RAL and RTV at concentrations reported in Table 1 (DRV-d_9 (I.S.) at 1000 ng/ml).

LLOQ) and to the highest QC level are shown in Fig. 2a and b, respectively, in the positive mode, using the Selected Reaction Monitoring Mode (SRM) and the proposed gradient program (Section 2.4). The respective retention times of antiretroviral drugs and the I.S. DRV-d_9 are reported in Table 2. The separation is satisfactory between 5.5 and 9.0 min for all considered analytes.

Though all antiretroviral drugs and I.S. were eluted within 10 min, a relatively prolonged rinsing step of 5 min at a flow-rate of 0.5 ml/min was introduced to eliminate some memory effect observed in the initial set-up of the analytical method. This rinsing step was followed by the column-conditioning step with the initial solvent composition (98/2 solvent A/solvent B) at a flow-rate of 0.4 ml/min (2.4 min) and 0.3 ml/min (2.5 min).

Fig. 3 shows the signals at all selected *m/z* transitions when a single solution containing all antiretroviral drugs and I.S. was continuously infused post-column directly into the MS/MS detector during the chromatographic analysis of six different blank plasma extracts. The signals at the *m/z* transition showed a remarkably similar pattern, with all traces being essentially superimposable. Even though no marked matrix effect (no drifts or shifts of the signals) was observed at the respective retention time of the antiretroviral drugs and I.S. peaks (shown in the chromatographic profile) in this perfusion experiment, some matrix effects were however found as reported in the experiments below (see Section 3.5).

The chromatograms in Fig. 4 were obtained from a patient under DRV (600 mg BID), ETV (200 mg BID), MVC (150 mg BID), RAL (400 mg BID) and RTV (100 mg BID). The blood sample was taken 1.5 h after last drug intake. The plasma levels measured were 5644 ng/ml, 603 ng/ml, 255 ng/ml, 251 ng/ml (=565 nM) and 563 ng/ml for DRV, ETV, MVC, RAL and RTV, respectively. Interestingly, the *m/z* transition selected for RAL showed in patients an additional peak at 5.07 min, eluted before RAL, which was hypothesized to be due to the in-source dissociation of raltegravir–glucuronide (RAL–gln), the only reported metabolite so far for RAL [18]. This peak identification was confirmed in a separate sample analysis by single ion monitoring at $[M\text{-}H]^-$ = 619, corresponding to the molecular weight of RAL–gln (MW 620), using the negative mode and a lower collision induced dissociation (CID) energy (=4 V). This is shown in the Fig. 5 on the chromatographic profile A of a sample taken 1.5 h after RAL intake from a patient under 400 mg BID. The chromatogram B in Fig. 5 shows the standard analysis of the same sample in the positive mode, using the SRM *m/z* transition 445.1 → 361.0 selected for RAL, confirming that the early peak co-elutes with RAL–gln (upper profile A). The intensity of the signal for RAL–gln in this patient was found to be approximately eight-fold higher than that measured for RAL. In fact, with the proposed method, large inter-individual differences in the extent of RAL glucuronidation have been observed so far, with individual RAL–gln/RAL ratios varying up to a 130-fold factor. Of importance, these inter-individual differences are not due to the variability in the efficiency of RAL–gln in-source fragmentation as RAL–gln/RAL ratios were found not to be significantly influenced by plasma matrix alteration (see Section 3.6). Some individuals have repeatedly RAL–gln plasma peak signals more than 10-fold higher than those measured for RAL. Those data, although preliminary, departs strikingly from the only study on RAL metabolism published so far in healthy volunteers after a single raltegravir dose, which reports that RAL–gln accounts for 30% of total RAL in plasma [18]. Whether the RAL–gln/RAL ratio constitutes a valid, clinically useful, marker of raltegravir glucuronidation is currently being evaluated as part of a controlled drug–drug and drug–gene interaction trial.

3.2. Internal standard and calibration curve

Ideally, deuterated analogues or homologues of the antiretroviral drugs would be the first-choice standards but these were not available to us. Several chemical compounds, unlikely found in patients but sharing some structural/chemical similarities with antiretroviral drugs, were evaluated as potential internal standards. Finally, DRV-d_9 was selected because it was used for the assay of darunavir, and had a negligible memory effect and, as a deuterated labeled compound, it is not present in patients.

For all antiretroviral drugs, calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactory described by either $1/x$ or $1/x^2$ weighted quadratic regression, or quadratic log–log regression, of the peak-area ratio of antiretroviral drug to I.S., *versus* the concentrations of the respective antiretroviral drugs in each standard sample.

Over the considered concentration range, regression coefficient r^2 of the calibration curves were always greater than 0.99 with back-calculated calibration samples within ±15% (± 20% at LLOQ).

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bag may not fully reflect the plasma matrix from HIV patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibration samples preparation would be impracticable and difficult to justify from an ethical point of view. For the sake of validation, the cross-validation has been performed between four series of the three levels of QC and two series of calibration samples analysed in duplicate (citrate *versus* EDTA). Regression analysis of the head-to-head comparison show that the anticoagulant does not influences significantly the results for raltegravir, darunavir, maraviroc and ritonavir (p = 0.98, 0.57, 0.97, and 0.97, respectively). For etravirine, a slightly significant (p = 0.04) bias of −5.9% was observed for EDTA samples analysed with citrate calibration. However, even if this observation was not spurious (simply due to test repetition without Bonferroni's correction), CI 95% values for this bias (−0.3 to −11.3%) are comprised within the ±15% allowance for analytical deviation and are unlikely to be of any notable clinical significance.

3.3. Precision and accuracy, LLOQ, LOD

Precision and accuracy determined with the QC samples are given in Table 3. The levels of control samples were selected to reflect the low, medium and high range of the calibration curves chosen to encompass the clinical range of concentrations found in patients' plasma. The mean intra-assay precision was similar over the entire concentration range and always less than 8.9%. Overall, the mean inter-day precision was good, with CV within 5.1–9.8%. The intra-assay and inter-assay deviations (bias) from the nominal concentrations of each analysed antiretroviral drug were comprised between −5.9 and +9.7%, and −3.3 to +5.1%, respectively.

The results of the determination of LLOQ and LOD of antiretroviral drugs in plasma are shown in Table 4. By analysing plasma samples spiked with decreasing concentrations, the lowest achievable LOD among the considered antiretroviral drugs was obtained for maraviroc at 125 pg/ml. The lowest LOQ was obtained for MVC at 0.5 ng/ml, corresponding to an amount of 5 pg of drug into the 10 μl injection volume. Overall, in the concentration ranges chosen for the establishment of the calibration curves (i.e. 12.5–5000 ng/ml, 2.5–1000 ng/ml, 25–10,000 ng/ml, 10–4000 ng/ml and 5–2000 ng/ml, for RAL, MVC, DRV, ETV and RTV respectively), the precision and accuracy of the lowest calibration sample were, for each antiretroviral drug, comprised within the ±20% limit recommended by the FDA [38], in line with the latest updates of the Washington and Arlington Conference reports [39].

Of note, the chosen ranges of calibration were selected initially to cover the *clinically relevant* range of antiretroviral drug concentrations presumably present in the plasma samples collected during

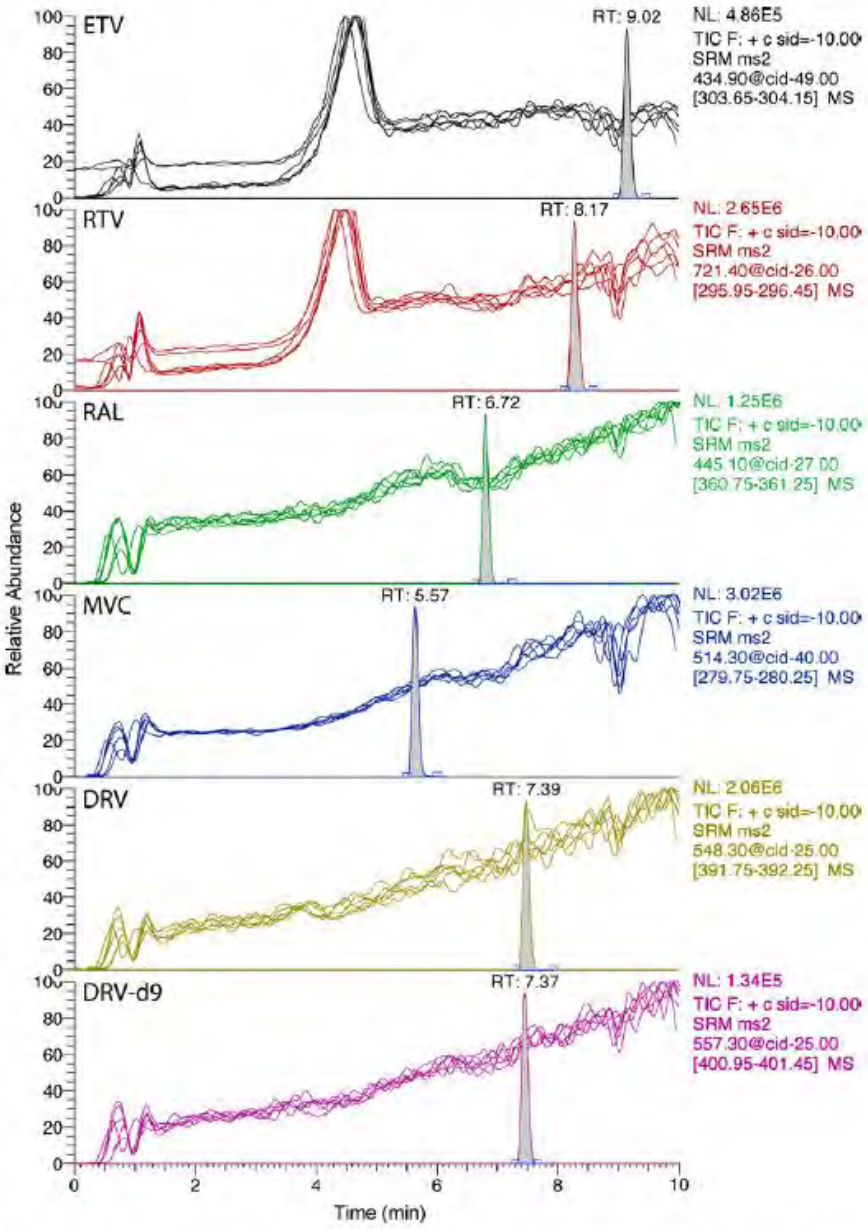

Fig. 3. Chromatogram of six blank plasma extracts with post-column infusion of a solution containing DRV, ETV, MVC, RAL, RTV and DRV-d_9 at 100 ng/ml. Chromatogram of a calibration sample is also shown.

the medical visit generally taken at random time after the last drug intake. Except in some cases of non-compliance to treatment, plasma drug levels encountered so far have been all lying above the lowest calibration levels. During the course of the method validation however, it was observed *a posteriori* that the performance of our tandem MS/MS detector enables us to attain lower detection and quantification limits, well below the clinically relevant range of antiretroviral drugs concentrations established during the validation procedure. These LLOQ and LOD attained were found to differ depending of the drug considered, and in the perspective of

Table 4
Limit of detection and lower limit of quantification of antiretroviral drugs.

	LOD (ng/ml)	LOQ		Accuracy at LOQ	Precision at LOQ
		(ng/ml)	(pg)	(bias%)	(CV%)
Darunavir	1.25	10.0	100	+0.02	4.1
Etravirine	2.5	10.0	100	−0.06	7.9
Maraviroc	0.125	0.5	5	−2.27	2.0
Raltegravir	1.25	7.8	78	−3.24	13.7
Ritonavir	1.0	1.0	10	−3.30	18.0

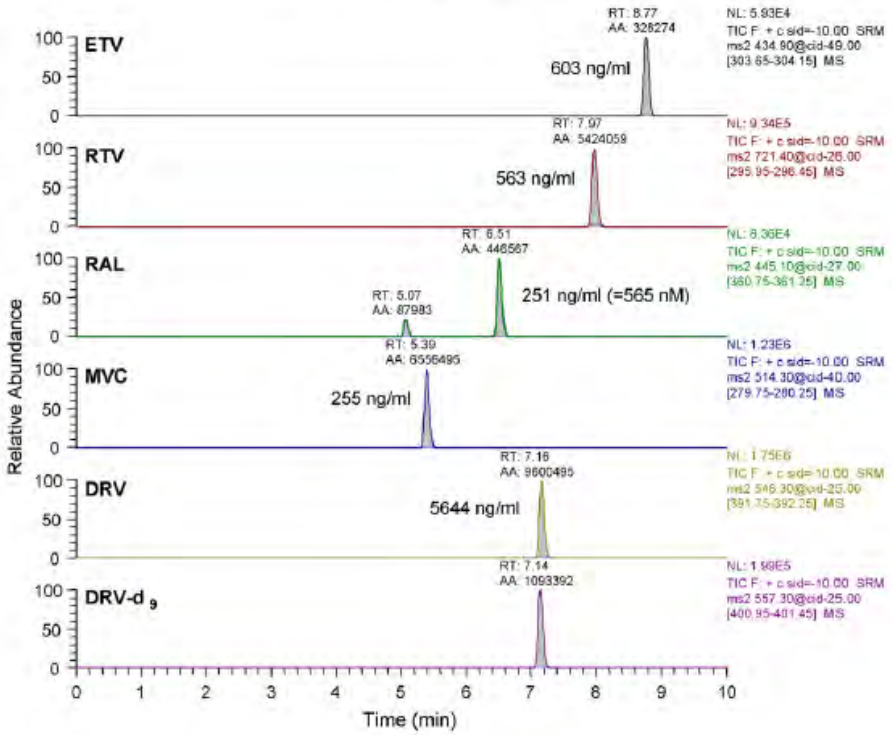

Fig. 4. Chromatogram of a plasma sample from a patient receiving DRV, ETV, MVC, RAL and RTV. The blood sample was taken 1.5 h after drug intake (details in the text).

this method validation, these results are reported in Table 4. However, for the sake of standardization and for the simplification of solution preparation, it was decided to keep the same – clinically relevant – range of calibration previously determined for all drugs, regardless of their LLOQ.

3.4. Stability of antiretroviral drugs

The stability of antiretroviral drugs in plasma QCs samples left at room temperature (RT) and at +4 °C was ascertained up to 48 h. The variation over time of each drug levels at 24 h and 48 h (Table 5) was mostly comprised within the ±15% of nominal concentrations (except for two values from ETV and RTV, where T24 exceed 15%) indicating that, taking into account the analytical variability, antiretroviral drugs can be considered generally stable.

Table 5 shows the variation of antiretroviral drug concentrations after one, two and three freeze–thaw cycles. For RAL, MVC, ETV and RTV, the variation was always less than 15%, indicating no significant loss of drug after up to three freeze–thaw cycles. For DRV, the variation after the third cycle at the highest concentrations tested slightly exceeded 15% but remained however below 20%.

Calibration and quality control samples were prepared in batches, distributed and stored at −20 °C in 4 ml polypropylene tubes for use up to 1 month in our routine antiretrovirals monitoring facility. No evidence of raltegravir, etravirine, darunavir and maraviroc decomposition was found during plasma samples storage in the freezer at −20 °C for at least 6 weeks. In the same conditions, a mean 10% decrease in ritonavir levels was noticeable but this variation always remains less than the ±15% allowance and should not affect to a clinically relevant extent ritonavir plasma levels determination. Of note, calibration samples subjected to the thermal viro-inactivation procedure (60 min at 60 °C in a waterbath) are not stable thereafter upon storage at −20 °C, indicating that calibration samples must be subjected to thermization procedure only on the day of analysis.

3.5. Matrix effect and recovery

Matrix effect was examined by the simultaneous post-column infusion of antiretroviral drugs and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma extracts from blood donors (see Section 3.1). Co-eluting matrix components may nevertheless reduce or enhance the ion intensity of analytes, possibly affecting the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all standard calibrations and quality control samples have been prepared by spiking drugs in human plasma.

The assessment of the *matrix effect* (Table 6) was quantified as the peak-area response of analytes added to blank plasma extracts (i.e. B, drugs added *after* extraction), expressed as the percentage of the response of standard solution of drugs directly injected onto the column (A) (ratio B/A, in Table 6). A value above or below 100% for the matrix effect indicates an ionisation enhancement or suppression, respectively. The results indicate that co-eluting plasma matrix components do not appear to affect significantly the ionisation of RAL, MVC, ETV and DRV (mean ratio B/A = 104%). In the spiking experiments, an ionisation enhancement of 27% was noticed for RTV but this effect was found remarkably stable not only at the three concentrations studied (128.4 ± 3.8%, 125.5 ± 9.8%, 126.6 ± 4.3%, for low, medium and high concentration, respectively) but also amongst the 6 different plasma matrices (variability <7.8%). Conversely, in the perfusion experiments, a small drop in the signal could be noticed at the retention time of ETV (Fig. 3). However, in the spiking experiments (Table 6) this showed to slightly affect the ME for the lower QC sample only (83%), with an acceptable reproducibility (CV 10%).

Appendix 2.1.

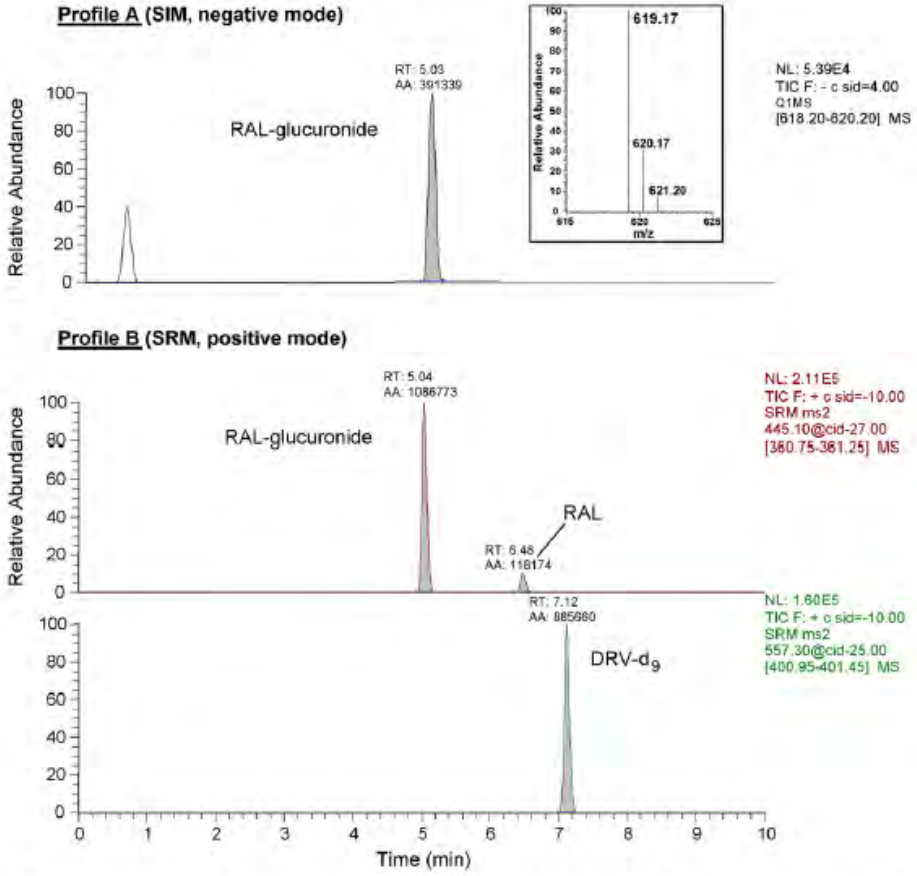

Fig. 5. Chromatogram of a plasma sample taken 1.5 h after drug intake from a patient receiving RAL 400 mg BID. This patient was also receiving EFV (600 mg QD) and LPV/RTV (600/150 mg BID).
Profile A: SIM in the negative mode at *m/z* $[M-H]^-$ → 619 selected for RAL–gln; *Offset*: MS spectra at the peak retention time for RAL–gln.
Profile B: standard SRM in the positive mode at *m/z* 445.1 → 361.0 selected for RAL; and transition for the I.S. DRV-d_9.

The *analytical recovery* was calculated in a similar way, but considering drugs response/I.S. ratio instead, before (C2) and after (B2) the extraction procedure (ratio C2/B2, in Table 6). Taking into account the I.S. response enables to correct for the occurrence of variation over time in the MS/MS spectrometer performance and injection volume. As indicated in Table 6, the extraction recovery for the I.S. was essentially 95%. The *analytical recoveries* achieved were always >96% for all antiretroviral drugs.

The *process efficiency* (i.e. overall recovery) given in Table 6 (column PE) was obtained as the peak-area response of analytes

Table 5
Stability of antiretroviral drugs in human plasma QC samples.

Drug		Darunavir			Etravirine			Maraviroc		
Nominal concentration (ng/ml)		250	1250	7500	100	500	3000	25	125	750
Room temperature	24 h	5.7	−6.6	7.0	2.2	−9.1	−9.3	3.3	2.2	0.3
	48 h	−2.1	3.2	−1.3	−13.5	−5.4	−9.7	2.1	8.2	−4.2
+4 °C	24 h	6.8	5.2	2.5	−24.0	−4.6	−6.6	0.7	5.6	−12.2
	48 h	−5.6	−9.0	−0.3	−11.5	−9.7	−0.2	−4.3	−7.4	−3.6
Freeze–thaw	cycle 1	−7.4	−4.7	−5.2	0.1	5.1	−0.5	−0.4	−2.8	−3.3
	cycle 2	−11.6	−7.2	−2.2	−14.9	3.6	1.5	−6.6	−6.7	−5.4
	cycle 3	−15.4	−9.3	5.8	−1.5	0.1	6.7	0.1	−4.0	2.3

Drug		Raltegravir			Ritonavir		
Nominal concentration (ng/ml)		125	625	3750	50	250	1500
Room temperature	24 h	2.9	4.9	0.7	−2.7	1.6	4.9
	48 h	−4.8	6.3	3.3	−5.6	1.5	−1.5
+4 °C	24 h	5.0	7.9	−2.7	−7.4	5.6	−19.2
	48 h	−14.4	−14.5	10.5	−14.2	−9.1	14.9
Freeze–thaw	cycle 1	8.0	−0.8	−6.8	−1.9	−4.7	2.8
	cycle 2	3.2	−4.7	−4.9	−1.7	−6.0	−8.5
	cycle 3	5.9	0.1	0.5	5.5	−3.3	2.4

Table 6
Matrix effect, extraction recovery, analysis recovery and process efficiency of antiretroviral drugs.

Compound	Nominal conc. (ng/ml)	Mean peak area			Mean peak area ratio		ME (%)	CV (%)	Ext RE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A (n = 3)	B (n = 6)	C (n = 6)	B2	C2	B/A		C/B		C2/B2			C/A	
Darunavir	250	333517	344809	323650	0.301	0.298	103.4	9.6	93.9	12.3	99.1	101.1	2.7	97.0	5.9
	1250	1574728	1680867	1658152	1.467	1.528	106.7	1.5	98.6	2.9	104.2			105.3	3.6
	7500	9207611	10457052	9916646	9.124	9.136	113.6	3.2	94.8	6.7	100.1			107.7	7.4
Etravirine	100	139647	116067	111041	0.101	0.102	83.1	10.0	95.7	11.9	101.0	100.5	4.1	79.5	4.4
	500	591132	583261	575618	0.509	0.530	98.7	4.8	98.7	4.4	104.2			97.4	5.9
	3000	2932636	3195373	2909228	2.788	2.680	106.8	3.5	91.0	7.2	96.1			97.2	6.0
Maraviroc	25	730295	768013	761102	0.670	0.701	105.2	4.4	99.1	5.0	104.6	106.1	1.2	104.2	3.2
	125	3492992	3618014	3662107	3.157	3.374	103.6	4.1	101.2	3.3	106.9			104.8	1.7
	750	17326261	18813088	19007243	16.414	17.512	108.6	2.9	101.0	4.5	106.7			109.7	3.8
Raltegravir	125	246764	236088	237709	0.206	0.219	95.7	8.1	100.7	12.0	106.3	104.2	2.7	96.3	4.7
	625	1141426	1211486	1206641	1.057	1.112	106.1	3.9	99.6	5.8	105.2			105.7	3.2
	3750	6419404	7187229	6882970	6.271	6.341	112.0	3.2	95.8	7.1	101.1			107.2	7.9
Ritonavir	50	597795	767365	738231	0.670	0.680	128.4	2.9	96.2	8.4	101.6	100.8	0.7	123.5	6.3
	250	2879597	3613948	3429056	3.153	3.159	125.5	7.8	94.9	3.0	100.2			119.1	5.7
	1500	15390132	19482061	18558090	16.998	17.058	126.6	3.4	95.3	6.0	100.6			120.6	7.2
Darunavir-d_9 (I.S.)	1000	1077729	1146132	1085390			106.3	3.5	94.7	5.0				100.7	4.0

A = Peak area of standard solutions without matrix and without extraction; B = Peak area of analytes spiked after extraction; C = Peak area of analytes spiked before extraction; B2 = Ratio of the peak area of the analyte and the I.S. spiked after extraction; C2 = Ratio of the peak area of the analyte and the I.S. spiked before extraction; ME = Matrix effect expressed as the ratio of the mean peak area of the analytes spiked after the extraction (B) to the mean peak area of the same standard solution without matrix (A) multiplied by 100; ext RE = Extraction procedure recovery calculated as the ratio of the mean peak area of the analytes spiked before extraction (C) to the mean peak area of the analytes spiked after extraction (B) multiplied by 100; Analysis RE = Analysis recovery calculated as the ratio of the mean peak area ratio of the analytes spiked before extraction (C2) to the mean peak area ratio of the analytes spiked after extraction (B2) multiplied by 100; PE = Process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (C) to the mean peak area of the same analyte standard (A) multiplied by 100.

spiked into plasma samples *before* the extraction procedure (C) – such as calibration and control samples – expressed as the percentage of the peak area of pure drug standard solution (A) directly injected into the column. This *process efficiency* takes into account the analytical recovery and the matrix effect: for example, DRV has a mean matrix-mediated ionisation enhancement close to 108% (Table 6, column ME), which combined with a mean extraction yield of 96% (Table 6, column ext RE) gives an process efficiency around 103%. Overall, these results indicate that even though no apparent matrix effect was observed in the infusion experiment (Fig. 3), matrix components do influence to some extent the overall process efficiency. This was especially noticeable for RTV at all QCs concentrations, and to a lesser extent ETV at the low QC level, requiring therefore the preparation of calibration and control samples in plasma matrix reflecting at best the composition of the samples to be analysed. Most importantly, it is not so much the matrix effect *per se* that must be reduced than its variability.

As shown in Table 6, the variability of the matrix effect of six different plasma matrices never exceeded 10.0%, demonstrating indeed that the proposed extraction procedure is able, if not to eliminate, at least to normalize and standardize the matrix effect.

3.6. Influence of plasma matrix variability on raltegravir–glucuronide/raltegravir ratios values

In patients' samples containing high amounts of RAL–gln, RAL–gln/RAL ratios values were not affected by sample alteration upon dilution 1:2 with six different sources of plasma. In patient samples with only small amounts of RAL–gln, some differences in RAL–gln/RAL ratios were noticed upon plasma matrix alteration but those differences were comparatively small, and of no clinical significance in the context of studies on patient's raltegravir glucuronidation rate. Overall, this indicates that any variability in plasma matrix composition does not affect, or only slightly RAL–gln/RAL ratios values. Importantly, the extremely high RAL–gln/RAL ratios observed in some individuals are thus not due to matrix effects that would have influenced the extent of RAL–gln in-source dissociation.

3.7. Dilution effect

After the five-fold dilution of the spiked plasma with antiretroviral drugs at a concentration exceeding by two-fold the high calibration level, the mean deviation (bias) from the expected concentrations was +1.0%, −0.3%, −2.2%, +4.0% and −6.6% for DRV, ETV, MVC, RAL and RTV, respectively. This indicates that plasma samples containing antiretroviral drugs above the high level of calibration can be adequately diluted with blank plasma prior to the LC–MS/MS analysis.

3.8. Clinical applications

This analytical method has been shown reliable and sensitive for monitoring plasma concentrations of RAL, MVC, DRV, ETV and RTV in patients as part of our routine TDM service and for clinical research projects done within the frame of the Swiss HIV Cohort Study.

Fig. 6 shows the plasma levels, plotted against time after drug intake, measured for DRV, ETV, RAL and MVC, given either separately, or in combination (see introduction), or all four drugs altogether, with an optimized background regimen. Available data show a very high inter-individual variability in RAL and ETV plasma levels in this experienced patient' population: these two drugs have therefore been prioritized for further investigations on the genetic

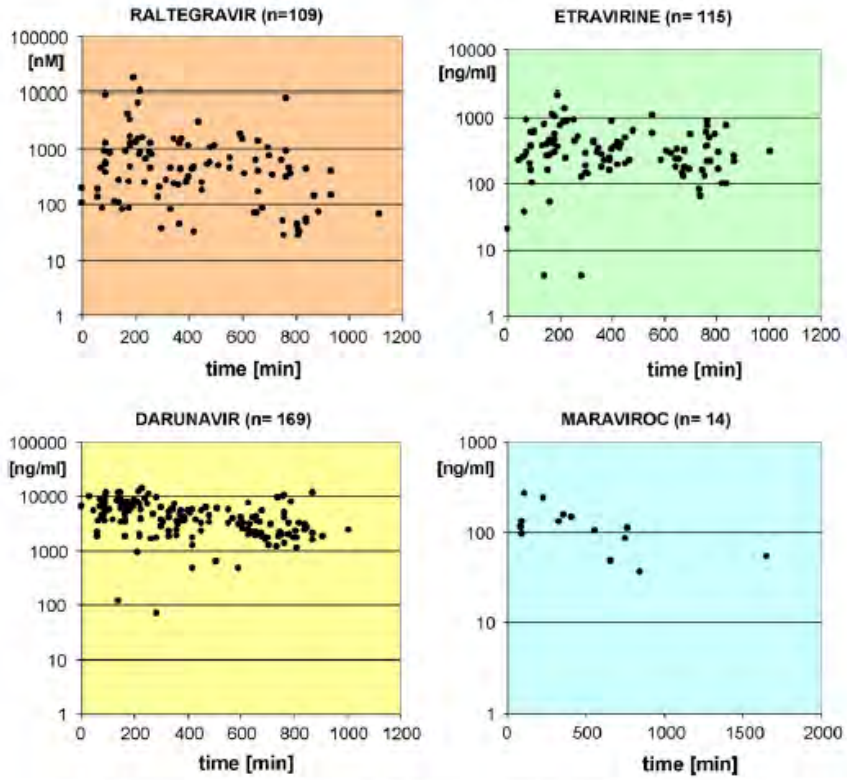

Fig. 6. Plasma levels plotted against time after drug intake of the latest four antiretroviral drugs.

and environmental factors that potentially influence their disposition. These studies are currently underway.

4. Conclusion

The reported and validated LC–MS/MS method provides a robust procedure for determining four novel antiretroviral drug concentrations in plasma, enabling their monitoring and a better understanding of their efficacy, toxicity and potential for drug interactions. The high sensitivity and specificity attained with LC–MS/MS enable the quantification of as little as 5–100 pg on-column, depending on the drug (Table 4), in an aliquot as low as 100 μl volume.

This is, to the best of our knowledge, the first validation report for a LC–MS/MS method for the simultaneous assay of antiretroviral agents targeted to four different steps of HIV replication, using the convenience of a single-step extraction and an elution gradient program allowing their quantification in the same analytical run.

The advent of novel drugs from current and new antiretroviral classes increases the complexity of use of antiretroviral drugs for effectively maintaining patients' health through the dual goal of long-term virological suppression with minimal toxicity. All the new agents have the potential for significant interactions with other antiretroviral medications, adding to the challenge for clinicians to devise long-term effective antiretroviral regimen combinations, while still having to deal with the complexity of previous PI- and NNRTI-based regimens and their associated drug interactions. With doubts hanging over the long-term effect of chronic CCR5 inhibition and the limited experience so far with the first integrase inhibitor raltegravir, a continuing monitoring seems essential for CCR5 antagonists and HIV integrase inhibitors. It is however too early to appreciate whether measuring these new antiretroviral drugs in plasma will add clinically useful information to the current TDM performed for PIs and NNRTIs in the follow-up of HIV patients.

This analytical method is suitable for monitoring new antiretroviral drugs, for suggesting lack of short-term compliance and for helping in the identification of drug interactions in new regimens, modifying the systemic disposition of drugs to an extent likely to impair virologic response.

Our method suitably answers the demands of clinicians for monitoring novel drugs administered in combination, often as salvage therapy, to heavily pre-treated patients, in whom exposure, tolerance and adherence assessments are critical issues. Further studies will determine its contribution to risk minimization and to therapy optimization.

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Protocole d'isolement de la fraction libre de médicaments antirétroviraux à partir de plasma non congelé

Produits chimiques :

- Solution standard de RTV-like 25 ng/mL dans MeOH/H_2O 50:50
- Solution Deconex à 0.5%

Equipements :

- Centrifugeuse Beckmann® Model J-6B
- Tubes Eppendorf 1.5 mL
- Pipettes de précision (1000 µl et 200 µl)
- Tubes Centrifree, Amicon bioseparation, Millipore®
- Centrifugeuse à angle fixe : Avanti® J-301 High Performance Centrifuge System, Beckmann®
- Vials en verre pour LC-MS

Normes de sécurité :

Les manipulations avec du matériel biologique potentiellement infectieux (sang, plasma) se font toujours avec des gants et des lunettes de protection, sous la hotte à flux laminaire.

Mode opératoire :

Séparation fraction libre :

- (Centrifuger les monovettes à 2000rpm (1850g) pendant 10 minutes (T 4°C, frein max).)
- Transférer 2x 750 µl de chaque plasma (duplicat) dans des Eppendorfs et centrifuger à 10'000rpm pendant 10 minutes (T 4°C) [pour séparer les composés parasites : gras, cellules restantes,…].
- Pendant ce temps, prélaver les tubes Centrifree en injectant 500 µl d'H_2O déminéralisée et en centrifugeant dans la centrifugeuse à angle fixe (BH19) (= ultrafiltration) : 25°C, 2000 g, 30 minutes. N.B. : si la machine note un problème de fermeture du couvercle, appuyer sur *CE* et *enter*.
- Vider la cupule de récupération de l'ultrafiltrat, la remettre en place et injecter 500 µl de plasma centrifugé dans le tube prélavé en maintenant le tube à 45° et sans toucher la membrane avec la pipette. Centrifuger (ultrafiltration) 15 minutes à 2000 g dans la centrifugeuse à angle fixe (T 25°C).
- Pendant ce temps, préparer les cupules de récupération finales : noter les cupules de la même manière que les tubes Centrifree, et les peser 1 à 1 en prenant soin de bien noter le poids.
- Récupérer les tubes et jeter les cupules contenant la première fraction d'ultrafiltrat (0-15 min). Les remplacer par celles qui ont été pesées. Remettre à centrifuger les tubes pendant 15 minutes (25°C, 2000 g). La deuxième ultrafiltration permet de récupérer environ 100 µl d'ultrafiltrat.
- Peser les cupules contenant l'ultrafiltrat, et y soustraire le poids de la cupule vide. Ajouter précautionneusement (c-à-d. en laissant couler le long de la paroi de la cupule) un volume de MeOH équivalent au poids de l'ultrafiltrat récupéré, et mélanger à l'aide de la pipette.
- Placer 100 µl du mélange UF/MeOH dans un vial d'injection, et y ajouter 20 µl de solution standard de RTV-like 25 ng/mL dans MeOH/H_2O 50:50. Fermer le vial et vortexer. Les vials peuvent être conservés au réfrigérateur (4°C) jusqu'à l'analyse.
- Le reste de l'ultrafiltrat est stocké à 4°C dans les cupules fermées (réserve).

Important : Après utilisation de la centrifugeuse à angle fixe, désinfecter l'intérieur avec une solution de Deconex 0.5%.

Analyse :

- Les fractions libres sont analysées par chromatographie liquide à haute performance couplée à la spectrométrie de masse en mode tandem (LC-MS/MS).

ORIGINAL ARTICLE

Determination of Unbound Antiretroviral Drug Concentrations by a Modified Ultrafiltration Method Reveals High Variability in the Free Fraction

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Abstract: Total plasma concentrations are used for therapeutic drug monitoring of antiretroviral drugs, whereas antiviral activity is expected to depend on unbound concentrations. The determination of free (unbound) concentrations by ultrafiltration may be flawed by the irreversible adsorption of many drugs onto the membrane filters and plastic components of the device. The authors describe a modified ultrafiltration method enabling the accurate measurement of unbound concentrations of 10 antiretroviral drugs by liquid chromatography–tandem mass spectroscopy, which circumvents the problem of loss by adsorption in the early ultrafiltration fractions. The method was applied to assess the variability of free fractions of antiretroviral drugs during routine therapeutic drug monitoring in 144 patients with HIV. In in vitro experiments, ultrafiltrate collected in four fractions (0–8, 8–16, 16–24, and 24–30 minutes) gave much lower and more variable free drug concentrations in the first ultrafiltrate fraction than in the last three fractions for lopinavir, nelfinavir, saquinavir, tipranavir, and efavirenz. In the last two fractions, free concentrations remained constant, indicating saturable adsorption. The adsorption was modest for indinavir, amprenavir, and ritonavir, and unnoticeable for atazanavir and nevirapine. Free fraction values obtained with this modified ultrafiltration method reveal substantial interindividual variability, suggesting that monitoring unbound antiretroviral drug concentrations may increase its clinical usefulness, especially for lopinavir, saquinavir, and efavirenz.

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Key Words: therapeutic drug monitoring, antiretroviral drugs, ultrafiltration, free concentrations, protein binding, LC-MS/MS

(*Ther Drug Monit* 2008;30:511–522)

INTRODUCTION

Drugs are present in two forms in plasma: bound to plasma proteins (mostly albumin and α-1-acid glycoprotein) and unbound (free). The unbound drug is usually considered the only form diffusing into tissues and penetrating into cells to exert its activity. Wide differences exist in the extent of binding of antiretroviral drugs (ARVs). HIV protease inhibitors (PIs) are mostly lipophilic weak basic molecules highly bound to plasma proteins (greater than 85% except indinavir, 60%), mainly to α-1-acid glycoprotein (AAG). The nonnucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine, weakly acidic, bind predominantly to albumin[1] (greater than 99% and 60%, respectively).

Total (bound + free) concentrations are currently determined for therapeutic drug monitoring (TDM) of ARVs.[2,3] A small change in the extent of protein binding of highly bound drugs results in a dramatic effect on the free fraction.[4,5] Diseases or infections can significantly alter the binding of drugs.[4–7] Pregnancy also affects protein binding.[4,8–13] Recently, phenotypic variants of AAG have been found to influence the clearance and distribution of some PIs.[14] For NNRTIs, total plasma concentrations correlate only modestly (efavirenz) or not at all (nevirapine) with cellular concentrations measured in peripheral blood mononuclear cells,[15] thus prompting investigations to identify whether variability in protein binding could explain this discrepancy.

Information on free drug concentrations and corresponding free fractions (free/total concentration ratio) could clarify the exposure to unbound ARVs induced by physiological, genetic, pathophysiological conditions, or pharmaceutical interactions and improve the contribution of TDM to treatment adjustment. Indeed, changes in free fraction can influence the interpretation of total drug measurement. For drugs of low extraction, a change in protein concentrations or binding affinity (albumin and/or α-1-acid glycoprotein) alters

total plasma concentrations, whereas free drug concentrations remain mostly unchanged.[16] Conversely, changes in protein binding for drugs of high extraction are not expected to alter total drug concentrations, whereas free drug concentrations are affected. In both cases, altered free fractions impact on total concentration–effect relationships and may compromise the correct interpretation of TDM results. Therefore, unbound drug concentration determination may provide a more accurate indicator of "effective" drug exposure and contribute to a better individualization of drug dosage regimens.[1,4,17,18]

Equilibrium dialysis and ultrafiltration are the methods most frequently used for free drug concentration determination.[4,5] Although equilibrium dialysis is considered the reference method for determining drug–protein binding, its results may still be biased by dilutional shifts in equilibrium caused by diffusion of free fraction into the dialysate, osmotic dilution of retentate, incomplete attainment of dialysis equilibrium, binding to buffer ions, and nonspecific drug adsorption onto the dialysis apparatus. However, the main drawback of equilibrium dialysis is the tedious, time-consuming procedure not suitable for routine use in clinical laboratories. By contrast, ultrafiltration is relatively quick and easily implemented for large numbers of samples. However, as for dialysis equilibrium, nonspecific binding to membranes and plastic components of ultrafiltration devices remains an issue potentially leading to underestimation of the unbound concentration.[4,5,19–21] Although ultrafiltration has been proposed for the determination of free concentrations of ARVs, irreversible adsorption onto device components was reported to underestimate the free fraction of nelfinavir and lopinavir by as much as 25%[17] and 50%,[22] respectively. On the other hand, adsorption seems negligible for amprenavir[23] and indinavir.[24] For saquinavir and tipranavir, the adsorption issue has not yet been addressed.

Such limitations probably explain why information on free concentrations of ARVs remains scarce. The few published investigations report small numbers of subjects receiving PIs[17,22–37] or NNRTIs[38,39] followed over one dosing interval. Free and total concentrations were found roughly correlated, but with substantial interindividual variability in free fraction.

We aimed at developing a method for the convenient and accurate measurement of unbound concentrations of ARVs (amprenavir [APV], atazanavir [ATV], indinavir [IDV], lopinavir [LPV], nelfinavir [NFV], ritonavir [RTV], saquinavir [SQV], tipranavir [TPV], efavirenz [EFV], and nevirapine [NVP]) using the method of ultrafiltration while circumventing the loss by adsorption. The method was applied to the assessment of interindividual variability in antiretroviral drug free fractions and to their correlation with albumin and AAG levels in patients with HIV undergoing routine TDM.

MATERIALS AND METHODS

Chemicals and Reagents

ATV was kindly provided by Bristol-Myers Squibb (Baar, Switzerland), APV by GlaxoSmithKline (Stevenhage, UK), EFV and IDV by Merck Sharp & Dohme-Chibret (Glattburg, Switzerland), LPV and RTV by Abbott (Abbott Park, IL), NFV and SQV by Roche (Basel, Switzerland), and NVP and TPV by Boehringer Ingelheim (Ridgefield, CT). The research compound A-86093 given by Abbott was used as the internal standard (I.S.) for the assay. Of note, deuterated analogs of most ARVs have been made available only recently (ie, d4-efavirenz, d8-lopinavir, d5-atazanavir, d5-nevirapine) (LGC Promochem, Molsheim, France) and should certainly be considered as the optimal I.S. at present.

Solvents (methanol [MeOH], acetonitrile; LiChrosolv® grade, 100% formic acid) were purchased from Fluka (Buchs, Switzerland) and ammonium acetate from Merck. Ultrapure water was obtained through a Milli-Q UF-Plus apparatus (Millipore Corporation, Burlington, MA).

Equipment

The high-performance liquid chromatography system involved a Rheos 2200 binary pump (Flux Instruments, Basel, Switzerland) equipped with an online degasser and a temperature-controlled 324 vial autosampler maintained at +10°C (CTC Analytics AG, Zwingen, Switzerland). The chromatographic system was coupled to a triple-stage quadrupole mass spectrometer (TSQ Quantum Ion Max) from Thermo Electron Corporation (Waltham, MA) equipped with an electrospray ionization interface and operated with the Xcalibur 1.1 software (Thermo Electron Corporation, San Jose, CA).

Liquid Chromatography–Mass Spectrometry Chromatographic Conditions

Chromatographic separations were performed on an Atlantis dC18 column (2.1 × 50 mm, 3 μm) (Waters, Milford, MA) in a thermostated column heater at 25°C (Croco-Cil; Clutzeau Info Laboratory Courbevoie, France).

Solution A consisted of acetonitrile with 0.1% formic acid. Solution B was 2 mM ammonium acetate containing 0.1% formic acid. Solution C consisted of acetonitrile with 1% formic acid. The mobile phase was delivered at 0.3 mL/min according to the following gradient elution program: 0 to 1 minute, 2% solution B → 30% B at 3.5 minutes, → 100% B at 10 minutes. The washing/re-equilibration step included an intensive rinsing (10 to 14 minutes with 100% of C at 0.5 mL/min, → 100% B at 15 minutes at 0.5 mL/min) followed by the initial solvent composition (2% solution B : 15.1 to 18 minutes at 0.4 mL/min and 18 to 20 minutes at 0.3 mL/min). For EFV analysis, solutions A and B were prepared without formic acid, enabling an enhanced sensitivity. The chromatographic separation of EFV and I.S. was obtained by a stepwise elution 0 to 1 minute, 2% solution B → 45% B at 2 minutes, → 45% B at 16.4 minutes followed by rinsing and re-equilibration steps up to 21 minutes. Quantification of ARVs was performed using selected reaction monitoring in the positive mode for all ARVs, EFV only being monitored in the negative mode. We used selected transitions and collision energies reported previously for PIs and NNRTIs[40] with some minor modifications. The *m/z* transition, collision energy (V), and tube lens used for TPV were 603.0 → 585.0, 25, and 77, respectively.

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Plasma and Ultrafiltrate Stock Matrix Preparation

Outdated blood transfusion bags were aliquoted in 50-mL aliquots into Falcon tubes and centrifuged without delay at 1850 g (3000 rpm) for 10 minutes at +4°C (Beckman Coulter Centrifuge, Model J6B, Fullerton, CA). The blank plasma was used directly for the in vitro experiments or, alternately, transferred into an Amicon Centricon Plus-20 Filter System (cutoff 30 kDa; Millipore Corporation, Bedford, MA) and submitted to ultrafiltration (1850 g, +4°C, 30 minutes) to obtain the blank ultrafiltrate pool for the preparation of calibration and quality control samples. Aliquots (100 μL) of ultrafiltrate were distributed in Eppendorf vials and stored at–20°C until use.

Ultrafiltrate Calibration and Quality Control Samples

Stock solutions at 1 mg/mL in MeOH for APV, ATV, EFV, NFV, RTV, SQV; at 1 mg/mL in MeOH/H_2O (50:50) for IDV and NVP; at 2 mg/mL in MeOH for LPV; and at 5 mg/mL in MeOH for TPV were diluted with MeOH for the preparation of working solutions at concentrations of 2 to 10,000 ng/mL. On the day of analysis, a 100-μL volume of each working solution was added to 100 μL ultrafiltrate to reach concentrations of 1, 5, 10, 50, 100, 500, and 1000 ng/mL for calibrators (5000 ng/mL for IDV and NVP) and concentrations of 2.5, 25, and 200 ng/mL for quality controls.

Total Calibration and Quality Control Samples

Blank plasma samples were spiked with stock solutions appropriately diluted to reach the clinically relevant total plasma concentrations for calibration: 50 to 10,000 ng/mL for APV, ATV, EFV, IDV, NFV, NVP, RTV, and SQV; 100 to 20,000 ng/mL for LPV; 1875 to 75,000 ng/mL for TPV; and for quality controls: 750, 3000, and 9000 ng/mL for APV, ATV, EFV, IDV, NFV, NVP, RTV, and SQV; 1500, 6000, and 18,000 ng/mL for LPV; and 5625, 22,500, and 67,500 ng/mL for TPV (total added volume MeOH 10% or less of the biologic sample volume in accordance with the recommendations on bioanalytical method validation.[41–43]

Calibration Curves

The calibration curve was measured with a series of calibration samples analyzed in duplicate at the beginning and at the end of the run.

Quantitative analysis of PIs and NNRTIs in ultrafiltrate and plasma were performed using the I.S. method.

Determination of Free Drug Concentrations

In Vitro Experiments With Test Samples

Before use, the ultrafiltration Amicon Centrifree Filter Systems (cutoff 30 kDa; Millipore Corporation) were conditioned with 500 μL of ultrapure water submitted to ultrafiltration (2000 g, 30 minutes, 26°C) in a fixed-edge, temperature-controlled centrifuge (Avanti J-30I High Performance Centrifuge System; Beckmann). In the in vitro experiments, blank plasma aliquots (500 μL) spiked with ARVs at two clinically relevant concentrations (750 and 2500 ng/mL for APV, ATV, EFV, IDV, LPV, NFV, NVP, RTV, and SQV; and 10,000 and 60,000 ng/mL for TPV) were placed in prewashed Centrifree ultrafiltration tubes and subjected to ultrafiltration at 2000g (3650 rpm). The ultrafiltrate was collected in four fractions (0–8, 8–16, 16–24, and 24–30 minutes) in preweighed plastic cups. Each ultrafiltrate fraction was weighed accurately and diluted 1:1 w/v with MeOH without delay. After the addition of 20 μL of I.S. solution (A-86093, 25 ng/mL in MeOH/H_2O 50:50) to a 100-μL aliquot of each ultrafiltrate fraction, the resulting samples were introduced into 500-μL high-performance liquid chromatography microvials. A 10-μL volume was used for the liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

For overall comparison, free drug concentrations were measured in ultrafiltrate from spiked blank plasma samples collected 1) as a single (0–30 min) collection, according to the recommendations of the manufacturer; or 2) the 15- to 30-minute only ultrafiltrate collection.

Application to Clinical Samples

The method described was applied to the measurement of free drug concentrations of antiretrovirals in patients for whom monitoring of total plasma concentrations was requested as part of their medical follow up.

Clinical samples were collected at random times after drug intake and processed in our laboratory as part of our routine TDM service. Samples were processed under a Class II Biohazard Protein Hood wearing nitrile gloves and long-sleeved lab coats. Briefly, blood samples (5 mL, EDTA) were collected as previously described and centrifuged at 1850 g (3000 rpm) for 10 minutes at +4°C (Beckmann Centrifuge, Model J6B). The plasma was divided into two aliquots, one for total plasma concentration monitoring and the other for free concentration determination. The first aliquot was heated at 60°C for 60 minutes in a thermostated water bath (Memmert WB 7, Schwabach, Germany) for viral inactivation.[44–47] The stability of ARVs has been demonstrated under those conditions.[48–51] Total concentrations were determined by LC-MS/MS after protein precipitation with acetonitrile using an adaptation of our previously reported method.[40] In brief, a 100-μL aliquot of patient plasma was mixed with 100 μL of I.S. solution (5 μg/mL for all ARVs, except for EFV, 80 ng/mL) and 600 μL of acetonitrile, vortexed, and sonicated for 10 minutes (Branson Ultrasonics Corporation, Danbury, CT). The mixture was again vortex-mixed and finally centrifuged at +4°C for 10 minutes at 20,000 g (14,000 rpm) on a Hettich Benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). An aliquot of the supernatant was diluted 1/75 (1/6 for EFV) with 20 mM ammonium acetate/MeOH (50:50). A 10-μL volume was injected into the LC-MS/MS instrument for the determination of total drug concentrations. The laboratory participates in an international external quality assurance program for the analysis of total concentrations of antiretroviral drugs (KKGT, Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie, Association for Quality Assessment in TDM and Clinical Toxicology, The Hague, The Netherlands).

The viral inactivation process was omitted in the second plasma aliquot to prevent alteration of protein binding. Free drug concentration was measured in duplicate in the second

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aliquot as follows: 500 μL of plasma was submitted to ultrafiltration and collected as two fractions (0–15 and 15–30 minutes) in preweighed plastic cups. The 15- to 30-minute ultrafiltrate collections were diluted 1:1 w/v with MeOH without delay. To 100-μL aliquots of each ultrafiltrate collection, 20 μL of I.S. solution (A-86093, 25 ng/mL in MeOH/H_2O 50:50) was added and the solution was analyzed by LC-MS/MS. The volume injected into the LC-MS/MS was 10 μL. The free fraction was calculated in each sample as the ratio of the free and the total concentration, expressed as a percentage.

Because patients with HIV were treated using a combined regimen with, for most, more than one drug subject to TDM (including RTV as a pharmacokinetic enhancer), the number of drug measurements exceeds the number of samples.

The study was purely observational and no dose recommendations were made on the results of free drug concentrations.

Plasma Albumin and α-1-acid Glycoprotein Concentrations

The albumin and AAG concentrations were measured using commercially available assays from Roche Diagnostics with colorimetric and immunoturbidimetric methods, respectively, carried out on a Roche Cobas Integra 400 (Roche Diagnostics, Rotkreuz, Switzerland).

Data Analysis

The correlation between total (C_{tot}) and free (C_{free}) concentrations and between free fractions and sampling times was assessed by standard linear regression analysis. The influence of AAG and albumin levels on total and free concentrations was examined by covariance analysis by incorporating AAG and albumin levels in the equation $C_{tot} = C_{free} + C_{free}$*[albumin] + C_{free}*[AAG] and examining the significance of their coefficient and their impact on the correlation coefficient.

Statistical significance was assigned at a P value less than 0.05.

RESULTS

Liquid Chromatography–Tandem Mass Spectrometry Analysis

Matrix Effect of Ultrafiltrate

A potential matrix effect was examined by the simultaneous postcolumn infusion of PIs/NNRTIs and I.S. into the MS/MS detector during the chromatographic analysis of six different batches of blank ultrafiltrates from healthy subjects diluted 1:1 with MeOH (Fig. 1). The profiles of the chosen m/z transitions during the course of chromatography were remarkably similar. No drifts or shifts of the selected transition signals were apparent at the retention time of PIs/NNRTIs and I.S. during the chromatography of those blank ultrafiltrate matrices. Coeluting matrix components may nevertheless reduce or enhance the ion intensity of analytes and affect the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all standard calibrations and quality control samples were prepared by spiking drugs in the same biologic matrix (ie, ultrafiltrate sample diluted 1:1 with MeOH).

Performance of the Free Drug Concentration Assays

Precision and accuracy of the LC-MS/MS assay were determined by analyzing the quality control samples at 2.5, 25, and 200 ng/mL selected to reflect low, medium, and high range of the calibration curves. The mean intraassay precision of the quality control samples was 5.0%, 4.1%, 7.9%, 5.1%, 4.1%, 5.4%, 5.2%, 9.8%, 4.8%, and 4.2% for APV, ATV, IDV, LPV, NFV, RTV, SQV, TPV, EFV, and NVP, respectively. Overall, the mean interday precision was good with average coefficients of variation within 4.3% to 9.3%. The intraassay deviation from the nominal concentrations of each analyzed antiretroviral drug was between –7.5% and 10.0% and the range of interday deviations was always lower than 7.4%. Mean percent coefficient of variation of duplicate determinations in patients (n = 296) (reproducibility) were 3%, 10%, 20%, 10%, 12%, 22%, 10%, 16%, and 5% for APV, ATV, LPV, NFV, RTV, SQV, TPV, EFV, and NVP, respectively.

In Vitro Experiments

Evolution of the Ultrafiltrate Concentration During Ultrafiltration of Test Samples

Free drug concentrations in the early 0- to 8-minute ultrafiltrate fraction were very low and highly variable (Fig. 2) with substantially lower free fractions than the later three fractions (8–16, 16–24, and 24–30 minutes) for LPV (mean, 0.89 versus 2.01%, $P < 0.05$), NFV (mean, 0.09 versus 0.52%, $P < 0.05$), SQV (mean, 0.98 versus 2.73%, $P < 0.05$), TPV (mean, 0.014 versus 0.072%, $P < 0.05$), and EFV (mean, 0.28 versus 1.17%, $P < 0.05$). In the last two fractions, the ultrafiltrate concentrations remained stable, indicating a saturable initial drug adsorption without influence on the accuracy of free drug concentration determination beyond 16 minutes. The adsorption phenomenon remained modest for IDV (mean, 45.0 versus 52.8%, nonsignificant), APV (mean, 18.1 versus 21.3%, nonsignificant), and RTV (mean, 6.6 versus 8.8%, nonsignificant) and unnoticeable for ATV (mean, 16.3 versus 18.6%, nonsignificant) and NVP (mean, 48.2 versus 45.7%, nonsignificant). The volume collected in each ultrafiltrate fraction was remarkably similar for all drugs (154 ± 15 μL [9.8%], 246 ± 18 μL [7.5%], 302 ± 19 μL [6.4%] and 337 ± 19 μL [5.5%] for fraction 0–8 minutes, 8–16 minutes, 16–24 minutes, and 24–30 minutes, respectively) indicating that the degree of drug protein binding and total and free drug concentrations in the samples had no influence on the ultrafiltrate flux across the membrane.

Comparison of Free Drug Concentrations Between Standard (0–30 Minutes) and Late-Only (15–30 Minutes) Ultrafiltrate Collection

The results presented in Table 1 indicate that the 15- to 30-minute fraction collection had higher free drug concentrations for all drugs, except for IDV and NVP. The free-fractions in Figure 2 are in general agreement with published values, notably those obtained by equilibrium dialysis.[1,17,32,34]

Of note, the in vitro experiments shown in Table 1 and in Figure 2 have been performed with different batches of blank plasma. The variability in plasma characteristics, notably albumin and AAG levels, explains the difference obtained in the free fractions of the antiretrovirals.

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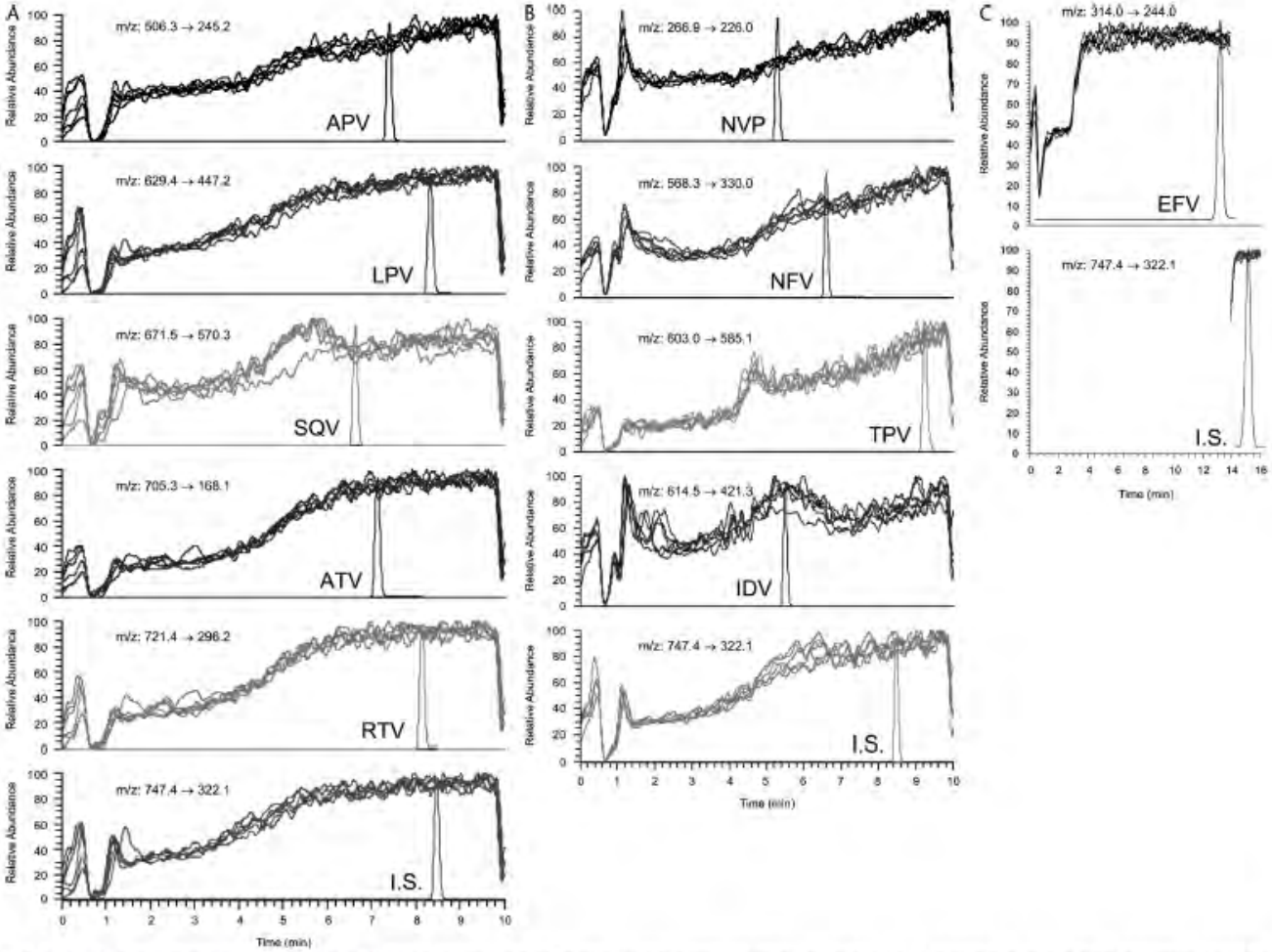

FIGURE 1. Chromatograms of six different blank ultrafiltrates with postcolumn infusion of a protease inhibitors/nonnucleoside reverse transcriptase inhibitors solution at 100 ng/mL of each drug and 100 ng/mL of internal standard (I.S.). Chromatographic profiles for the selected m/z transitions for: A) amprenavir, lopinavir, saquinavir, atazanavir, ritonavir, and I.S.; B) nevirapine, nelfinavir, tipranavir, indinavir, and I.S.; C) efavirenz and I.S.

Drugs Concentrations in Patients

Total and free drug concentrations were measured in 151 samples taken at random time after drug intake in 144 patients undergoing routine TDM for APV (n = 4), ATV (n = 45), EFV (n = 51), LPV (n = 56), NFV (n = 3), NVP (n = 20), RTV (n = 97), SQV (n = 17), and TPV (n = 3) (296 determinations). The range of plasma total and free concentrations, the mean free fractions with their range, and variability (percent coefficient of variation) are shown in Table 2.

Free Fractions in Patients

Figure 3 shows the free/total plasma concentration ratio (free fraction) plotted against the sampling time after drug intake. No significant time-related trend is observed in the free fraction over the dosing interval (slopes between -0.319 and $0.042\ h^{-1}$, $r < 0.3$). The small number of measurements available for APV, NFV, and TPV precludes any conclusion.

Correlation Between Free and Total Antiretroviral Drug Concentrations

Figure 4 shows the correlation between free and total antiretroviral drug concentrations. The correlation coefficient is 0.91 for ATV and NVP, 0.70 and 0.83 for LPV and RTV, and 0.59 and 0.50 for SQV and EFV, respectively.

Effect of α-1-acid Glycoprotein and Albumin Levels on Free Fractions

AAG and albumin levels were available for 146 samples and ranged from 0.21 to 2.71 g/L and 26.2 to 60.3 g/L, respectively. The free fractions of antiretrovirals plotted against AAG and albumin levels are shown in Figure 5. The influence of AAG and albumin levels on the relationship between free and total concentrations was also examined for ATV, LPV, RTV, EFV, and NVP. Incorporating α-1-acid glycoprotein and albumin plasma levels in the linear regression model resulted in clear improvement of the correlation for LPV (r values: 0.70 → 0.92), mostly as a result

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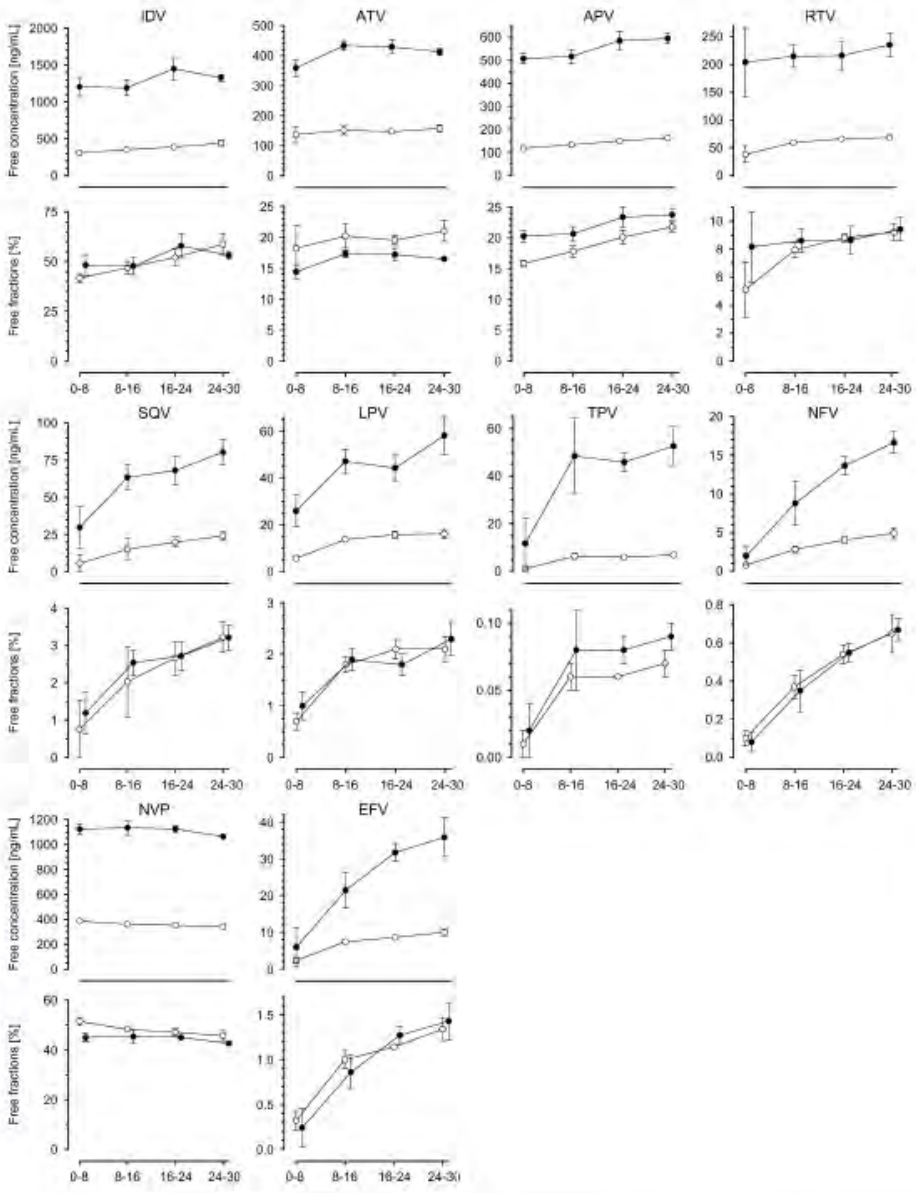

FIGURE 2. Free concentrations and corresponding free fractions in 0–8 minute, 8–16 minutes, 16–24 minute, and 24–30 minute fractions. Open and closed circles correspond to total plasma concentrations of 750 ng/mL and 2500 ng/mL, respectively, for amprenavir, atazanavir, lopinavir, indinavir, ritonavir, saquinavir, efavirenz, and nevirapine (10,000 ng/mL and 60,000 ng/mL for tipranavir).

of the influence from AAG ($P < 0.001$) and to some extent albumin ($P = 0.049$). The improvement was moderate for RTV (0.83 → 0.89) and ATV (0.91 → 0.93), attributable solely to AAG ($P \leq 0.001$). In contrast, neither albumin nor AAG levels influenced the correlation between free and total concentrations for NVP and EFV.

DISCUSSION

Determination of Free Drug Concentrations by Ultrafiltration

The validity of ultrafiltration for the accurate measurement of free drug concentrations has been questioned regularly, because it is not performed under equilibrium conditions and protein concentrates in the retained phase as ultrafiltration proceeds, this effect being even greater near the membrane where plasma proteins accumulate. Following the law of mass action, protein binding of the drug is expected to increase and free drug to decrease progressively during ultrafiltration. This was not observed in our study. Free drug concentrations remained remarkably stable for IDV and NVP, both drugs for which adsorption is not an issue. This confirms earlier observations reported during the determination of phenytoin or valproate free concentrations.[52–54] In fact, free protein and protein-bound drug are equally impermeable through the ultrafiltration membrane; they concentrate at the same rate and, therefore, their ratio remains constant. Consequently, the progressive increase in protein concentration does not alter the drug–protein binding equilibrium and

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TABLE 1. Comparison of Free Drug Concentrations With Standard (0-30 Minutes) Versus Late (15–30) Minutes Only) Ultrafiltrate Collection

Drug	Total Concentration [ng/mL]	Free fraction [%]		Absolute Difference	Relative Loss in Free Fraction With the 0–30 Min Method
		0–30 min	15–30 Min		
Amprenavir	750	7.3	7.9	0.6	7.5%
	2500	8.3	9.0	0.8	8.4%
Atazanavir	750	14.1	19.6	5.5	28.0%
	2500	13.2	20.1	6.9	34.1%
Indinavir	750	57.4	57.6	0.2	0.4%
	2500	53.0	50.2	−2.8	−5.6%
Lopinavir	750	0.35	0.45	0.10	22.2%
	2500	0.56	0.95	0.39	40.7%
Nelfinavir	750	0.10	0.34	0.24	69.6%
	2500	0.12	0.35	0.23	66.8%
Ritonavir	750	1.6	1.9	0.3	16.4%
	2500	1.8	2.0	0.2	8.4%
Saquinavir	750	1.7	2.9	1.2	41.3%
	2500	1.5	2.7	1.2	43.6%
Tipranavir	10000	0.08	0.09	0.01	13.2%
	60000	0.11	0.13	0.02	15.6%
Efavirenz	750	0.44	1.10	0.66	59.8%
	2500	0.55	1.10	0.55	49.7%
Nevirapine	750	56.0	54.8	−1.2	−2.3%
	2500	60.2	51.6	−8.6	−16.8%

the concentration of free drug in the ultrafiltrate as verified in the rigorous mathematical model proposed by Sophianopoulos et al.[55]

Loss of Free Drug Resulting From Adsorption

During early steps of the method development, we observed an important loss of free antiretroviral drug in the membrane filter and a lack of reproducibility unless the Centrifree tubes were first washed before plasma ultrafiltration. Therefore, we compared ultrafiltration performance after prewashing the devices with phosphate buffer, ammonium acetate (5 mM, 10 mM, 20 mM) and, according to the manufacturer's instructions, with purified water and NaOH (0.1 N) to reduce interferences between filtered drugs and trace amounts of glycerin in the membrane. The best recovery was obtained, for all drugs, by prewashing the tubes with pure water.

Using test-spiked plasma samples with our modified procedure, we noted a marked, albeit saturable adsorption of some ARVs in the first ultrafiltration fraction (0–8 minutes) and to a lesser extent in the second (8–16 minutes) without further effect on free drug concentration beyond 16 minutes of ultrafiltration.

This nonspecific adsorption phenomenon was especially pronounced for EFV, LPV, NFV, SQV, and TPV. Such a mechanism was considered responsible for the underestimation of NFV and LPV free concentrations.[17,22] Loss of EFV during ultrafiltration has also been recognized previously.[38] For SQV and TPV, the adsorption issue had never been addressed, but our results suggest that ultrafiltration may underestimate actual free concentrations. Lower free drug concentrations in the first ultrafiltration fraction have also been reported with the Centrifree system for other drugs such as

TABLE 2. Antiretroviral Drugs in Blood Sample of Patients With HIV: Range of Total and Free Plasma Concentrations, Mean Free Fractions, Their Range and Variability (percent CV%)

	APV	ATV	EFV	LPV	NFV	NVP	RTV	SQV	TPV
Total plasma concentration (ng/mL)	303–6450	168–2940	487–9827	677–23767	266–7266	2306–9976	27–2251	65–4216	11180–38735
Free plasma concentration (ng/mL)	11.5–324.2	8.1–291.7	2.1–30.3	4.2–209.2	0.3–6.6	1015–4836	0.1–22.8	0.2–25.8	0.8–32.2
Mean free fraction (%)	4.1	9.6	0.40	0.73	0.09	46.6	0.82	0.76	0.042
Range free fraction (minimum–maximum)	3.2–5.0	4.8–15.0	0.14–0.75	0.14–1.68	0.09–0.10	31.9–69.2	0.15–1.81	0.22–1.57	0.007–0.083
Variability free fraction (CV%)	18	25	36	51	5	20	48	59	92
N	4	45	51	56	3	20	97	17	3

CV%, percent coefficient of variation; APV, amprenavir; ATV, atazanavir; EFV, efavirenz; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir; TPV, tipranavir.

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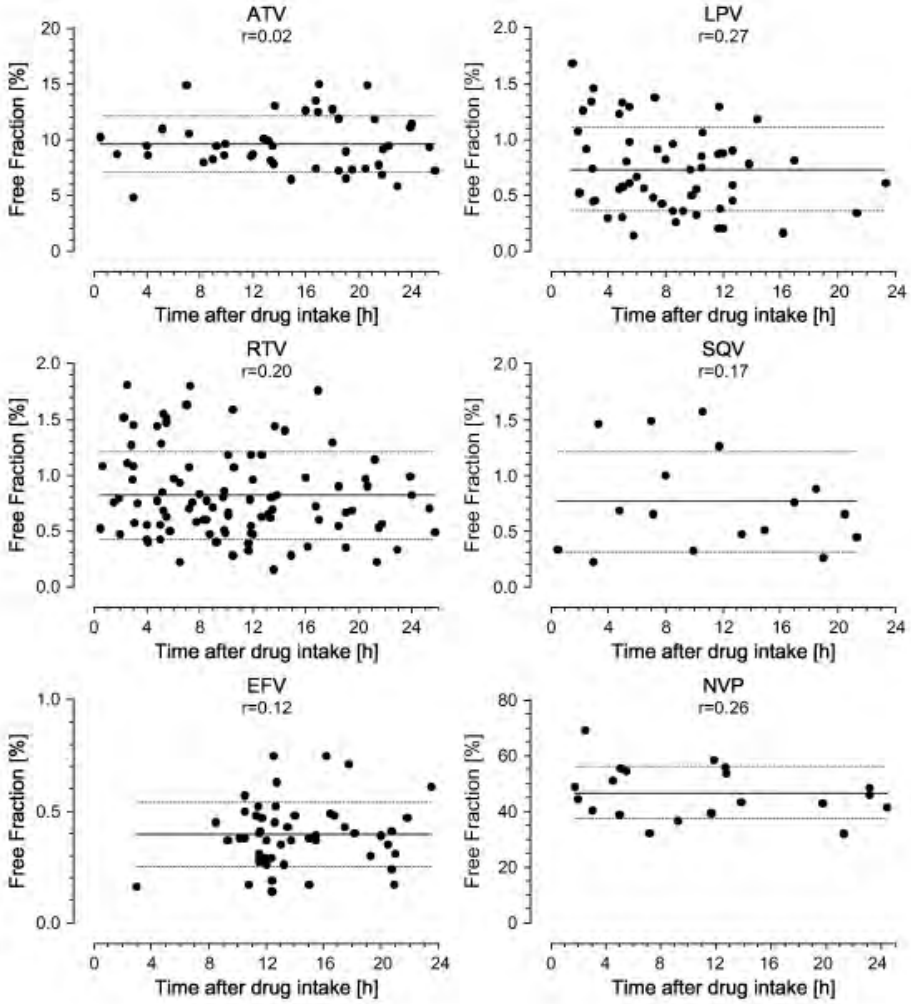

FIGURE 3. Free fractions over the dosing interval. Full lines and scattered lines represent mean free fraction and 95% confidence intervals, respectively.

phenytoin and valproate.[52,54] Globally, however, the importance of early adsorption during the ultrafiltration procedure and its effect on free concentrations has attracted little attention. Adsorption may take place not only onto the ultrafiltration membrane, but also onto the plastic walls of collecting cups. The adsorption on the membrane seems saturable, so it can be circumvented by selectively collecting the ultrafiltrate eluted after 15 minutes (Fig. 2). The loss of free lipophilic drugs from the ultrafiltrate aqueous environment onto the plastic walls of the cup can be minimized by the rapid addition of MeOH 1:1 w/v to ultrafiltrates in accordance with previous studies reporting for instance no adsorption of NFV to containers in solutions containing 35% v/v or more of acetonitrile or methanol in water.[17]

The volume of ultrafiltrate collected in each fraction was similar for all ARVs, regardless of their respective protein binding, and had no influence on free drug determination, in line with the observations of McMillin.[53] This indicates that the increase in free drug concentration observed for some drugs during the course of plasma ultrafiltration is not the result of a change in membrane efficiency.

Of note, antiretroviral free concentrations determined in our in vitro experiments were found to be slightly higher that those in patients, a finding in line with previously reported in vitro free fraction values determined in spiked samples.[23,31,34] Besides intrinsic plasma characteristics, the presence of an organic solvent (MeOH) in the spiked plasma samples may slightly alter the antiretroviral drug–protein binding equilibrium either by increasing free drug solubility or by modifying plasma proteins conformation. Thus, when in vitro ultrafiltration experiments are considered, efforts should be made to limit the percentage of organic solvent added for the preparation of spiked samples as much as possible.

Average Values and Interindividual Variability of Free Fractions

Our data allowed us to assess the interindividual variability of free fractions for the major antiretroviral drugs in patients undergoing routine TDM. In this population, free fraction values for ATV, LPV, and RTV were higher than those determined in 10 patients included in our previous ATV-LPV/r

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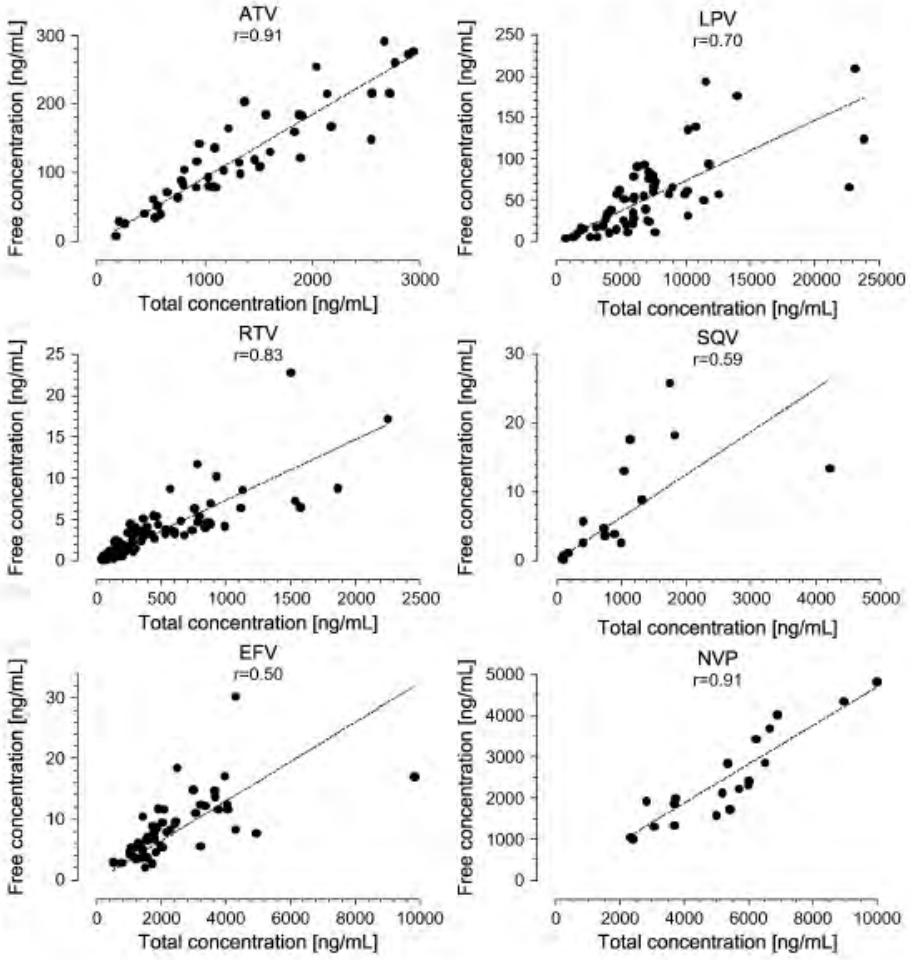

FIGURE 4. Total versus free drug concentrations.

pharmacokinetic interaction study,[25] possibly because loss by adsorption was not fully appreciated at that time. In addition, differences in patient characteristics and concomitant drugs might be involved. The comparison of our free fraction values with available data from the literature indicates good agreement. For example, the mean LPV free fraction of 0.73 ± 0.37% found in our 54 patients is similar to the reported value of 0.92% assessed by Boffito et al in 23 patients using the AUC_{free}/AUC_{total} ratio.[22] The range of RTV free fraction in our study (0.15–1.8%) also compares well with the range of 0.8% to 1.6% reported in patients receiving LPV/r,[30] but with a higher variability (48%). The range of free fraction values observed in the small number of patients on SQV (0.22–1.57%) is slightly lower than the 0.55% to 2.7% and 1.1% to 2.4% reported for SQV, with or without RTV, respectively.[28] In the only two available reports on NNRTIs, Almond et al[38,39] have measured median EFV and NVP free fractions (range) of 0.6% (0.4–1.5%) and 31.9% (16.2–63.3%), respectively, in 10 patients with HIV; in our study, the corresponding values were 0.40% (0.14–0.75%) and 47% (32–69%). Our results confirm, on the other hand, the substantial variability in the free fraction previously found for NVP. Finally, the APV free fraction of 4.1% observed in our four patients are toward the lower limit of the wide range reported in 10 patients (4.4–20%).[23] To the best of our knowledge, no data have yet been published on the free fraction of TPV, which we found to average 0.042% in three patients (range, 0.007–0.083%).

Despite the large variability observed in the free fractions, both our results and other available data show that free concentrations fluctuate in proportion with total concentrations, and no time-related trend was seen in free fractions over a dosing interval for SQV, IDV, and EFV,[28,38] although some small changes in free fraction have been reported for IDV[27] and for LPV.[22] Some changes in ATV, LPV, and RTV free fractions over a dosing interval were also noted in 10 patients in our previous ATV-LPV/r pharmacokinetic interactions study.[25] However, such fluctuations remained quantitatively small. Overall, the protein binding equilibrium can be considered established over a relatively short time, indicating that the dispersion of free fraction values found in the present study do not primary reflect a change over a dosing interval, but mainly the interindividual variability. For all drugs studied, free to total concentration ratios versus time after last dose intake show a homogenous distribution over the entire dosing interval.

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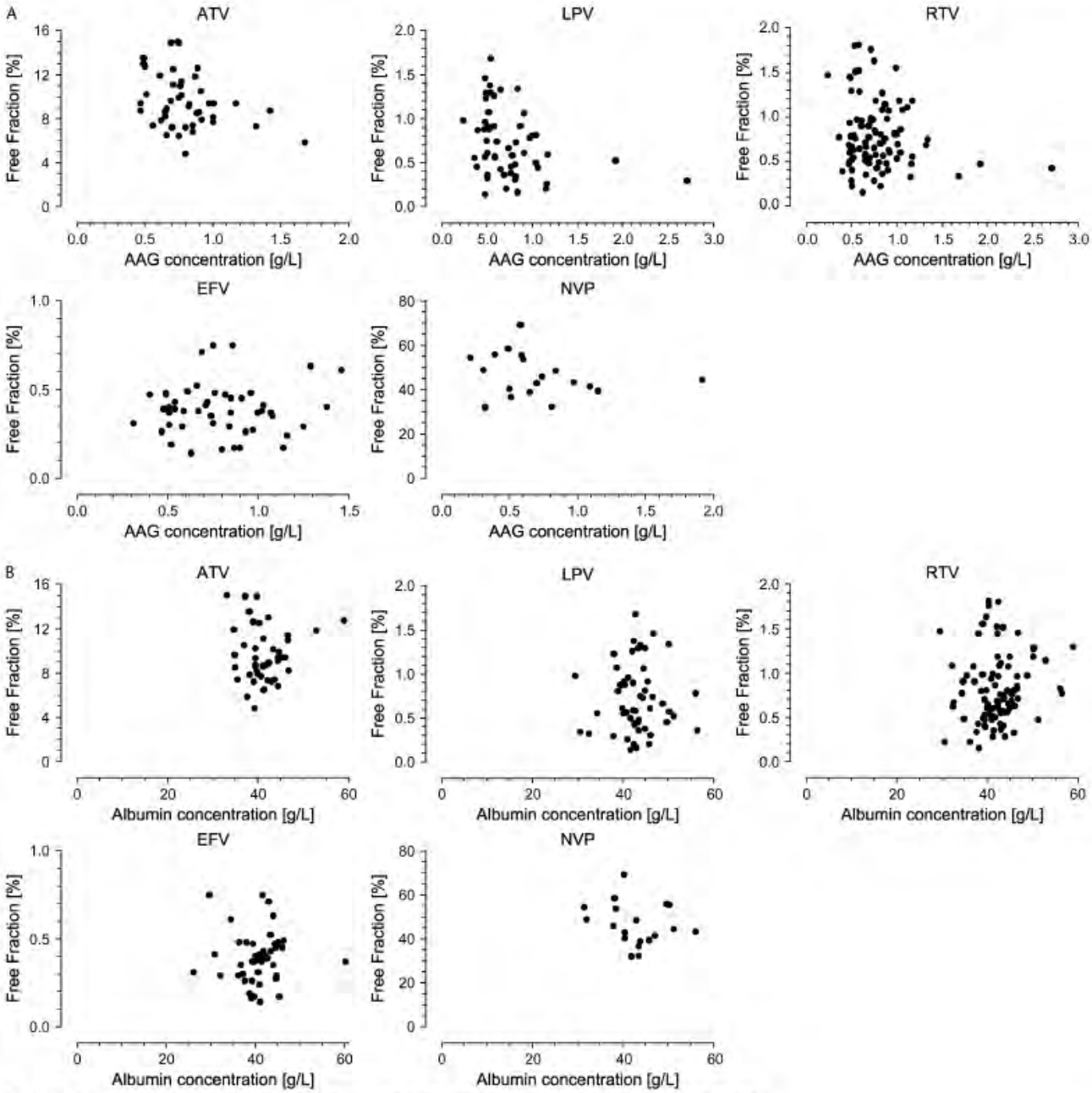

FIGURE 5. Free fraction plotted against α-1-acid glycoprotein (A) and albumin (B) levels.

Correlation Between Free and Total Antiretroviral Drug Concentrations

The correlation can be considered strong for ATV and NVP, moderate for LPV and RTV, and poor for SQV and EFV. For APV, NFV, and TPV, free and total plasma concentrations seem well correlated, but the small number of data for these PIs imposes caution in the interpretation of such observations.

The good correlation between free and total drug concentrations for ATV in 45 patients extends and confirms our observation made previously in 10 patients.[25] The modest correlation between free and total concentrations for LPV ($r = 0.70$), although confirming our previous findings,[25] seems to differ from the only other report published, showing a good correlation between free and total areas under the concentration–time curve ($r = 0.93$).[22] Interestingly, the correlation in our study was much improved ($r = 0.70 \rightarrow r = 0.92$) by integrating plasma AAG and albumin levels into the model, confirming that these proteins, especially AAG, influence LPV

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clearance. We also found a poor correlation between free and total concentrations for SQV (r = 0.59), in disagreement with a previous report.[28] Finally, the correlation between free and total concentrations for NVP and EFV have, to the best of our knowledge, not been reported elsewhere.

Effect of α-1-acid Glycoprotein and Albumin Plasma Levels on Antiretroviral Free Fraction

Although studies on this issue have been advocated,[1,56] this is the first exploration of the influence of AAG and albumin on free and total drug concentrations in patients with HIV. The study of the factors affecting drug–protein binding is important, because protein binding represents a potential variable for the accurate interpretation of TDM data. For example, in pregnancy, plasma volume expansion creates a significant degree of dilutional hypoalbuminemia, probably decreasing the binding capacity of plasma albumin, with a consequential increase in drug free fraction for some drugs. If the increased free fraction leads to a higher free concentration, pregnant women would be expected to have exaggerated drug effects. For most agents (low extraction drugs), changes in protein binding are not likely to alter free concentration profiles and pharmacologic effects, because unbound drug determines both the effect and the biotransformation rate and clearance. Clinicians who do not have access to unbound drug measurement may erroneously interpret low total concentrations as indicative of low exposure and inappropriately increase the doses with a subsequent risk of toxicity.[16]

Clinical Significance of Free Concentrations of Antiretroviral Drug

Only if total concentrations correlate well with free concentrations can monitoring of total plasma concentrations be considered a valid surrogate for the exposure to pharmacologically active drug. The fact that for some ARVs, free and total concentrations are only correlated to a limited extent deserves further investigation in terms of clinical consequences. Suboptimal clinical response or unexpected toxicity can be observed in patients with apparently appropriate total plasma levels; this might be explained in part by free drug concentrations markedly departing from usual values. Facing an unexpected clinical response, knowledge of free/total plasma concentration changes may give useful information. Of note, the Genotypic Inhibitory Quotient or Phenotypic Inhibitory Quotient have been proposed as a monitoring index for PI-based regimens in the guidelines published recently for TDM of antiretroviral drugs.[57] In fact, the protein-adjusted IC_{50} proposed at present in Phenotypic Inhibitory Quotient calculations assumes a constant drug–protein binding without attention to the substantial interindividual variability in drug–protein binding.

CONCLUSION

The procedure proposed here for the drugs determination of free concentrations of antiretroviral circumvents drug loss resulting from adsorption of some ARVs onto the membrane and plastic components of the ultrafiltration devices. This method may even be considered for other drugs extensively bound to proteins and adsorbed on ultrafiltration devices.

Applied to samples drawn for routine TDM, this procedure reveals a substantial variability in the free fractions of some ARVs, suggesting free rather than total drug concentrations are important, adding an additional level of complexity to the interpretation of total antiretroviral drug concentrations. Because total concentrations may only imperfectly reflect the free, pharmacologically active concentration, pharmacokinetic/pharmacodynamic relationships may have been obscured by the variability in protein concentration and drug binding. For highly bound drugs (ie, greater than 98%, LPV, EFV, SQV), small variations in free drug fraction may have a profound influence on overall exposure to the pharmacologically active drug.

With no controlled studies comparing TDM based on total versus free drug measurement, it is premature to recommend performing TDM using free drug concentrations. In special populations or situations such as pregnancy, altered proteins level, liver insufficiency, or nephrotic syndrome, such an approach may help in clinical decision-making.

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ETUDE DES CONCENTRATIONS LIBRES ET TOTALES DES MEDICAMENTS ANTIRETROVIRAUX PENDANT LA GROSSESSE

FEUILLE D'INFORMATION POUR LES PATIENTES

Concerne : Mme .....*(Nom, Prénom de la patiente)*

Information donnée par : ..... *(Investigateur)*

Date : ..... Lieu : .....

Madame,

Vous recevez actuellement, ou allez recevoir, conformément aux directives suisses, un traitement antiviral durant votre grossesse. Ce traitement vise avant tout à bloquer au maximum la réplication du virus afin d'éviter l'apparition de résistance et maintenir les défenses immunitaires. Le traitement se justifie durant la grossesse car il a été démontré qu'une diminution de la charge virale maternelle s'accompagne d'une réduction du risque de transmission à l'enfant. Même si l'indication au traitement est clairement admise, il reste néanmoins des points à étudier quant aux modalités de prescription et à la posologie des médicaments en cours de grossesse. La grossesse s'accompagne en effet de changements physiologiques pouvant modifier la manière dont les médicaments sont absorbés, transformés et éliminés.

Nous vous proposons de participer à une étude dont l'objectif est de mesurer les concentrations *totales* et les concentrations *libres* de médicaments dans le sang lors de la grossesse. Pour cela, un prélèvement sanguin de 5.5 ml sera effectué à 5 reprises lors de vos rendez-vous habituels avec le gynécologue/infectiologue : un au 1er trimestre, un au 2ème trimestre, un au 3ème trimestre, un au début de l'accouchement, et le dernier 2-3 mois après l'accouchement. La très petite quantité de sang prélevé pour ces examens ne comportent pas de risques pour vous et votre enfant. Ces échantillons de sang ne seront pas conservés après la fin de l'étude.

Si vous acceptez de participer, il vous sera demandé lors de chaque visite, de préciser les heures de prise des dernières doses de médicaments. La personne responsable de l'étude se chargera également de relever des informations à partir des dossiers médicaux et infirmiers (compte rendu des visites et résultats de laboratoire). Il est entendu que toutes les données personnelles récoltées sont anonymisées et gardées strictement confidentielles. Toute publication des résultats se fera de manière totalement anonyme.

Votre participation à ce protocole ne vous apportera pas de bénéfices directs. Vous êtes parfaitement libre d'accepter ou non de participer à cette étude. Si vous décider d'y participer, vous demeurez libre d'interrompre votre participation à tout moment, sans avoir à vous justifier. Un refus de participation n'affectera en rien vos relations avec l'équipe soignante ni n'influencera le traitement ou les soins qui vous seront prodigués durant votre grossesse ou ultérieurement.

Dr. Begoña MARTINEZ DE TEJADA Dr. Laurent DECOSTERD

Vers. 28.03.2006

Centre Hospitalier Universitaire Vaudois

ETUDE DES CONCENTRATIONS LIBRES ET TOTALES DES ANTIRETROVIRAUX PENDANT LA GROSSESSE

FEUILLE DE CONSENTEMENT

La personne soussignée

- certifie avoir été informée sur les objectifs et le déroulement de l'étude ci-dessus.
- affirme avoir lu attentivement et compris les informations écrites fournies en annexe dont elle a reçu copie, informations à propos desquelles elle a pu poser toutes les questions qu'elle souhaitait.
- atteste qu'un temps de réflexion suffisant lui a été accordé.
- a été informée que la confidentialité des données recueillies pendant l'étude lui est garantie.

La personne soussignée accepte de son plein gré et sur une base volontaire de prendre part à cette étude et demeure libre de se retirer de l'étude à tout moment pour n'importe quelle raison et sans préjudice. En cas de retrait de l'étude, les données recueillies dans le cadre de cette étude ne seront pas utilisées.

Nom et prénom de la patiente : .....

Signature : .....

Lieu et date : .....

Nom et coordonnées du médecin : .....

Signature : .....

Lieu et date : .....

Vers. 28.03.2006

Centre Hospitalier Universitaire Vaudois

ETUDE DES CONCENTRATIONS LIBRES ET TOTALES DES MEDICAMENTS ANTIRETROVIRAUX

FEUILLE D'INFORMATION

Concerne : Mme .....*(Nom, Prénom de la patiente)*

Information donnée par : ..... *(Investigateur)*

Date : ..... Lieu : .....

Madame,

Vous recevez actuellement un traitement antiviral. Ce traitement vise avant tout à bloquer au maximum la réplication du virus afin d'éviter l'apparition de résistance et maintenir les défenses immunitaires.

Nous nous proposons d'étudier l'influence des modifications physiologiques induites par une grossesse sur le devenir des médicaments, afin de pouvoir optimiser le traitement des femmes enceintes présentant la même affection que vous. Dans ce cadre, il serait utile de disposer également de données dans un groupe contrôle le plus similaire possible.

C'est la raison pour laquelle nous vous proposons de participer à une étude dont l'objectif est de mesurer les concentrations *totales* et les concentrations *libres* de médicaments dans le sang. Pour cela, un prélèvement sanguin de 5.5 ml sera effectué lors de l'un de vos rendez-vous habituels. La très petite quantité de sang prélevé pour ce examen ne comporte pas de risques pour vous. Ces échantillons de sang ne seront pas conservés après la fin de l'étude.

Si vous acceptez de participer, il vous sera demandé lors de la visite, de préciser l'heure de prise des dernières doses de médicaments. La personne responsable de l'étude se chargera également de relever des informations à partir de votre dossier médical et infirmier (compte rendu de la visite et résultats de laboratoire). Il est entendu que toutes les données personnelles récoltées sont anonymisées et gardées strictement confidentielles. Toute publication des résultats se fera de manière totalement anonyme.

Votre participation à ce protocole ne vous apportera pas de bénéfices directs. Vous êtes parfaitement libre d'accepter ou non de participer à cette étude. Si vous décider d'y participer, vous demeurez libre d'interrompre votre participation à tout moment, sans avoir à vous justifier. Un refus de participation n'affectera en rien vos relations avec l'équipe soignante ni n'influencera le traitement ou les soins qui vous seront prodigués.

Dr. Begoña MARTINEZ DE TEJADA Dr. Laurent DECOSTERD

Vers. 28.03.2006

Centre Hospitalier Universitaire Vaudois

ETUDE DES CONCENTRATIONS LIBRES ET TOTALES DES ANTIRETROVIRAUX

FEUILLE DE CONSENTEMENT

La personne soussignée

- certifie avoir été informée sur les objectifs et le déroulement de l'étude ci-dessus.
- affirme avoir lu attentivement et compris les informations écrites fournies en annexe dont elle a reçu copie, informations à propos desquelles elle a pu poser toutes les questions qu'elle souhaitait.
- atteste qu'un temps de réflexion suffisant lui a été accordé.
- a été informée que la confidentialité des données recueillies pendant l'étude lui est garantie.

La personne soussignée accepte de son plein gré et sur une base volontaire de prendre part à cette étude et demeure libre de se retirer de l'étude à tout moment pour n'importe quelle raison et sans préjudice. En cas de retrait de l'étude, les données recueillies dans le cadre de cette étude ne seront pas utilisées.

Nom et prénom de la patiente : .....

Signature : .....

Lieu et date : .....

Nom et coordonnées du médecin : .....

Signature : .....

Lieu et date : .....

Vers. 28.03.2006

Centre Hospitalier Universitaire Vaudois

ETUDE DES CONCENTRATIONS LIBRES ET TOTALES DES MEDICAMENTS ANTIRETROVIRAUX PENDANT LA GROSSESSE:

UNE ÉTUDE PROSPECTIVE EN COLLABORATION AVEC LA SWISS HIV COHORT STUDY (SHCS)

Ce formulaire doit être joint à l'échantillon sanguin prélevé dans un **tube** de **5.5. ml sur EDTA-K** et envoyé par poste en utilisant la protection recommandée pour le transport de matériel biologique à l'adresse suivante :
Laboratoire de Pharmacologie et Toxicologie cliniques, CHUV, BH18-218, 1011 LAUSANNE
Pour les questions, contacter s.v.p. Aurélie FAYET, pharmacienne : Aurelie.Fayet@chuv.ch, 021 314 42 98

Patiente :

Nom *(2ème lettre)* ▨ ☐ **Prénom** *(2ème lettre)* ▨ ☐

Date de naissance : _____ **Ethnie :** _____

Numéro cohorte (SHCS) ☐☐☐☐☐

Poids (avant la grossesse) .....kg

Hypertension :
☐ chronique ☐ gestationnelle ☐ pré-éclampsie

☐ Présent	☐ Absent

Consommation	Oui	Non	Quantité
- tabac	☐	☐	.....cigarettes / jour
- alcool	☐	☐	.....verres / jour

Demandeur :

Nom du médecin : .....
Adresse : .....
.....
.....
Téléphone : .....

Date du PROCHAIN rendez-vous :

.....

Prélèvement : **Date :** / / **Heure exacte :** .....h.....min **Poids** (à la visite) .....kg

☐ **Grossesse :**
Age gestationnel : ☐ semaines ☐ jours Dernière menstruation : / /

☐ **Le jour de l'accouchement**

☐ **Cordon ombilical**

☐ **Post-partum** Date de l'accouchement : / /

☐ **Patiente non-enceinte (contrôle)**

1. Médicaments antirétroviraux

Indiquer ici seulement les médicaments à doser : *inhibiteurs de protéase et/ou névirapine.*

Substance	Dose (mg)	Nombre de prise par jour	Date de début du traitement, ou dernier changement de posologie	Date et heure exacte de la dernière prise de médicament	
				/ /	.....h.....min
				/ /	.....h.....min
				/ /	.....h.....min

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2. Autres médicaments actuels

Indiquer ici *tous les autres médicaments* (y.c. les autres antirétroviraux non indiqués en 1).

Médicaments	Dose (mg)	Nombre de prise par jour

Médicaments	Dose (mg)	Nombre de prise par jour

3. Effets indésirables observés

	absent	présent	Grade* (1- 4)
1. Nausées	☐	☐	.....
2. Vomissements	☐	☐	.....
3. Autres :	☐	☐	.....
4. Toxicité :	☐	☐	.....

* Grade 1 : toxicité légère
Grade 2 : toxicité modérée
Grade 3 : toxicité sévère
Grade 4 : toxicité grave

Commentaires :

4. Informations cliniques

Si pas encore disponibles lors de la visite, les communiquer ultérieurement à Aurélie FAYET, responsable des analyses (voir recto).

	Valeurs	Date
1. CD4	.....cell/mm^3	/ /
2. Virémie	.....copies/mL	/ /
3. Créatinine sérique	..... µmol/L	/ /
4. ASAT	.....U/L	/ /
5. ALAT	.....U/L	/ /

	Valeurs	Date
6. Hémoglobine	.....g/L	/ /
7. Leucocytes	.....G/L	/ /
8. Neutrophiles	..... %	/ /
9. Plaquettes	.....G/L	/ /

Commentaires :

Formulaire d'avis du CE

Sur mandat de la Commission centrale d'éthique de la recherche sur l'être humain, le Comité départemental d'éthique de Maternité-Pédiatrie, dans la composition détaillée en page 2) a procédé en date du 25 janvier 2006 à une évaluation approfondie du projet de recherche désigné ci-après.

Désignation du projet de recherche : matped 06-003 n° de réf. CER : **06-011**

Concentrations libres et totales des médicaments antirétroviraux pendant la grossesse : étude prospective en collaboration avec la Swiss Mother & Child HIV cohort study.

Investigateur

Nom, prénom, titre:	Dr B. Martinez de Tejada
Fonction:	Médecin cheffe de clinique
Adresse:	Service d'obstétrique - Maternité – HUG – 1211 Genève 14

Le comité d'éthique base son appréciation sur l'ensemble des documents joints au "formulaire de base pour la soumission d'un projet de recherche biomédicale" du 11 janvier 2006 en annexe.

☒ procédure ordinaire ☐ procédure simplifiée ☐ évaluation ultérieure

Le comité d'éthique arrête l'**avis** suivant:

☒ **A Avis positif**
☐ **C Avis conditionnel** (v. page 2 et suiv.)
Evaluation ultérieure par le comité d'éthique nécessaire ☐
Information écrite au comité d'éthique suffisante ☐
☐ **D Avis négatif motivé (et explication pour réexamen)** (v. page 2 et suiv.)
☐ **E Avis justifié de ne pas entrer en matière** (v. page 2 et suiv.)

L'avis s'applique également aux autres investigateurs mentionnés dans la "demande d'évaluation", travaillant dans des sites de recherche relevant du champ de compétence du CE.

Recommandations

(extensible)

Conditions

(extensible)

Motifs de l'avis négatif et explication pour réexamen

(extensible)

Motifs de l'avis de ne pas entrer en matière

(extensible)

N° de réf. CE : 06-011 (matped 06-003) page 2

Composition du comité d'éthique

L'avis du comité d'éthique ayant siégé dans sa composition détaillée ci-après est valable, le quorum étant atteint (art. 32 de l'Ordonnance sur les essais cliniques de produits thérapeutiques du 17 octobre 2001).

					participe à l'avis	
	Nom, prénom	**Profession, titre**	**m**	**f**	**oui**	**non**
Présidence	BOULVAIN M.	Médecin-gynécologie, Obstétrique HUG	☒	☐	☒	☐
Vice-Prés.	POSFAY BARBE K.	Médecin-Pédiatre, infectiologue	☐	☒	☒	☐
Membres	BISCHOF P.	Biochimiste, labo hormonologie	☒	☐	☒	☐
	DUPERREX O.	Médecin-pédiatre, expert en biométrie	☒	☐	☒	☐
	GAUTHEY M.	Médecin-pédiatre, pédopsychiatre	☐	☒	☒	☐
	KERN Ch.	Médecin, anesthésiologie	☒	☐	☒	☐
	MORRIS M.	Médecin, généticien	☒	☐	☐	☒
	PARIS V.	Médecin pédiatre, méd. adolescence	☐	☒	☒	☐
	IMSAND E.	Juriste	☐	☒	☐	☒
	MARTINET M.	Infirmière-enseignante	☐	☒	☒	☐
	MOREL L.	Infirmière en gynécologie	☐	☒	☐	☒
	VITRY E.	Sage-femme, clinique obstétrique	☐	☒	☒	☐
	FLORES P.	Membre externe, représ. Intérêts parents	☐	☒	☒	☐
	LOMBARD B.	Membre externe, représ. intérêts parents	☐	☒	☒	☐
	RAPIN P.	Médecin gynécologue, en Ville	☒	☐	☒	☐
	REY J.P.	Médecin-pédiatre en Ville	☒	☐	☒	☐

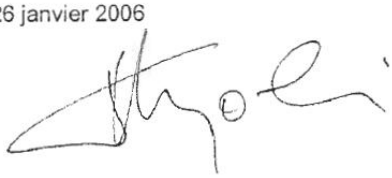
Pour mémoire : **Obligation de l'investigateur**

- les produits testés et de comparaison (médicaments et dispositifs médicaux) doivent être fabriqués, évalués et utilisés conformément aux règles de l'art visant à en garantir la qualité et la sécurité.
- Devoir de signaler:
  a) immédiatement tout événement indésirable grave (serious adverse events)
  b) toute information devenant disponible au cours de l'essai et ayant des conséquences directes pour la sécurité des sujets et la poursuite de l'essai
  c) modification du protocole
  d) fin ou arrêt prématuré de l'essai
- Rapport intermédiaire : une fois par année
- Notification d'essais de médicaments auprès de Swissmedic et de dispositifs médicaux auprès de l'OFSP (en cas d'étude sponsorisée, cette tâche incombe au promoteur)
- Rapport final

Le Comité d'éthique :

Lieu, date : Genève, le 26 janvier 2006 Nom : Dr Michel BOULVAIN

Signature :



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Faculté de biologie
et de médecine

COMMISSION D'ETHIQUE
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Prof. Jérôme Biollaz
Médecin chef
Division de Pharmacologie clinique
CHUV
1011 Lausanne

Lausanne, le 4 avril 2006

Avis de la Commission d'Ethique de la recherche clinique

Monsieur et cher Collègue,

Lors de sa séance du **13 février 2006**, la Commission d'Ethique de la recherche clinique, Sous-Commission I (composition détaillée en page 4) a procédé à une évaluation approfondie du projet de recherche désigné ci-après :

Protocole 30/06 : Concentrations libres et totales des médicaments antirétroviraux pendant la grossesse : Etude prospective en collaboration avec la Swiss Mother & Child HIV Cohort Study

Investigateur :

Prof. Jérôme Biollaz
Médecin chef
Division de Pharmacologie clinique
CHUV
1011 Lausanne

Copie : Mme Anne-Sylvie Fontannaz, Pharmacien cantonal, Service de la santé publique, Rue Cité-Devant 11, 1014 Lausanne

No de réf. de la Commission d'Ethique :30/06 2

La Commission d'éthique de la recherche clinique, Sous-Commission I, base son appréciation sur les documents soumis les 2 février 2006 et 28 mars 2006, soit :

- Votre lettre du 2 février 2006 et votre lettre du 28 mars 2006
- Acceptation de la Commission d'éthique de Genève du 26 janvier 2005
- Protocole en français du 2 février 2006
- Feuille d'information pour les femmes enceintes, version du 28.03.2006
- Feuille de consentement pour les femmes enceintes, version du 28.03.2006
- Feuille d'information pour les femmes groupe contrôle, version du 28.03.2006
- Feuille de consentement pour les femmes groupe contrôle, version du 28.03.2006

☒ procédure ordinaire ☐ procédure simplifiée ☐ évaluation ultérieure

La Commission d'Ethique arrête l'**avis** suivant :

☒ **A Avis positif**

☐ **B Avis positif assorti de recommandations** (v. page 3 et suiv.)
Information écrite à la Commission d'éthique suffisante ☐

☐ **C Avis conditionnel** (v. page 3 et suiv.)
Evaluation ultérieure par la Commission d'éthique nécessaire ☐
Information écrite à la Commission d'éthique suffisante ☐

☐ **D Avis négatif motivé (et explication pour réexamen)** (v. page 3 et suiv.)

☐ **E Avis justifié de ne pas entrer en matière** (v. page 3 et suiv.)

L'avis s'applique également aux autres investigateurs mentionnés dans la demande d'évaluation, travaillant dans des sites de recherche relevant du champ de compétence de la Commission d'Ethique de la recherche clinique.

Pour mémoire : **Obligations de l'investigateur**

Les produits testés et de comparaison (médicaments et dispositifs médicaux) doivent être fabriqués, évalués et utilisés conformément aux règles de l'art visant à en garantir la qualité et la sécurité.

Devoir de signaler :
a) immédiatement tout événement indésirable grave (serious adverse events)
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Rapport intermédiaire : une fois par année

Notification d'essais de médicaments auprès de Swissmedic et de dispositifs médicaux auprès de l'OFSP (en cas d'étude sponsorisée, cette tâche incombe au promoteur)

Rapport final

Emolument : Fr. 200.—

La Commission d'éthique, Sous-Commission I :

Prof. M. Burnier
Président

Centre Hospitalier Universitaire Vaudois

DÉPARTEMENT DE MEDECINE — LABORATOIRE de PHARMACOLOGIE CLINIQUE

ETUDE SHCS # 571 - TAUX PLASMATIQUE EFAVIRENZ OU LOPINAVIR

Division de pharmacologie clinique (PCL)
Laboratoires BH18 - 218
Centre Hospitalier Universitaire Vaudois
1011 LAUSANNE

Labo TEL: 021 / 314 42 71 - FAX : 021 / 314 42 88

Taux des médicaments: 021/314 42 98
E-mail: **Aurelie.Fayet@chuv.ch**

PATIENT

Nom: .....
Prénom: .....
Sexe: .....
Date de naissance complète: .....
Adresse complète: .....
.....
N° postal / Localité: .....
.....
Votre référence: .....
Facture au: ☐ patient ☐ demandeur

Etiquette code barres pour le CHUV

Copie (s) des résultats à:
Nom du médecin: .....
Adresse: .....
Bip / Tél: .....

Demandeur:
Nom du médecin: .....
Adresse: .....
Bip / Tél: .....

INFORMATIONS INDISPENSABLES

Traitement antirétroviral: indiquer au dos tous les antirétroviraux que le patient a déjà reçus sous «ancien» et préciser la posologie pour les «actuels».

DATE ET HEURE DU PRÉLÈVEMENT : **Date:** | | | | | | | | | **Heure:** | | | | |

ATTENTION ! ETUDE SHCS # 571 : NE PAS FACTURER

DONNÉES PHARMACOLOGIQUES : cf VERSO
Patient inclus dans l'Etude suisse de Cohorte HIV: ☐ oui ☐ non N°Cohorte/SHCS_ID

ECHANTILLON POUR:

☐ BASELINE	Date du prél.
☐ APRES ADAPTATION 1	Date du prél.
☐ APRES ADAPTATION 2	Date du prél.

Transmis en pharmacologie le : ..... Visa : .....
(à remplir par le laboratoire)

N° LID/CHUV

verso ➔

Centre Hospitalier Universitaire Vaudois

DÉPARTEMENT DE MEDECINE — DEPARTEMENT DE PATHOLOGIE ET DE MEDECINE DE LABORATOIRE

☐ SHCS # 571 : TAUX PLASMATIQUE EFAVIRENZ OU LOPINAVIR

Condition requise: traitement stable depuis au moins 1 mois; Prélèvement : **5 ml** de sang **EDTA-K** ou plasma EDTA-K congelé

Prélèvement sanguin *au moins*: 8h après la dernière prise d'efavirenz; 4h après la prise de lopinavir

DATE ET HEURE DU PRÉLÈVEMENT : Date: | Heure:

Demande d'interprétation clinique de la mesure du taux de médicament : ☐ oui (par défaut) ☐ non

NRTIs

	Ancien	Actuel
Retrovir® (zidovudine)	☐	☐
3TC® (lamivudine)	☐	☐
Zerit® (stavudine)	☐	☐
Videx® (didanosine)	☐	☐
Ziagen® (abacavir)	☐	☐
Viread® (tenofovir)	☐	☐
Emtriva® (emtricitabine)	☐	☐
Combivir® (AZT+3TC)	☐	☐
Trizivir® (AZT+3TC+ABC)	☐	☐
Kivexa® (ABC+3TC)	☐	☐
Truvada® (emtricitabine + tenofovir)	☐	☐
Autres préciser :		
.....	☐	☐

INHIB. FUSION

Fuzéon® (enfuvirtide)	☐	☐
Autres préciser :		
.....	☐	☐

POSOLOGIE : **DATE et HEURE *RÉELLE* de la DERNIÈRE PRISE :**

PIs

	Ancien	Actuel							
Invirase® (saquinavir)	☐	☐		mg		x/j	Date:		Heure:
Norvir® (ritonavir)	☐	☐		mg		x/j	Date:		Heure:
Viracept® (nelfinavir)	☐	☐		mg		x/j	Date:		Heure:
Crixivan® (indinavir)	☐	☐		mg		x/j	Date:		Heure:
Agenerase® (amprenavir)	☐	☐		mg		x/j	Date:		Heure:
Telzir® (fosamprenavir)	☐	☐		mg		x/j	Date:		Heure:
Kaletra® (lopinavir)	☐	☐		mg		x/j	Date:		Heure:
Reyataz® (atazanavir)	☐	☐		mg		x/j	Date:		Heure:
Aptivus® (tipranavir)	☐	☐		mg		x/j	Date:		Heure:
Prezista® (darunavir)	☐	☐		mg		x/j	Date:		Heure:
.....	☐	☐		mg		x/j	Date:		Heure:

NNRTIs

Stocrin® (efavirenz)	☐	☐		mg		x/j	Date:		Heure:
Viramune® (nevirapine)	☐	☐		mg		x/j	Date:		Heure:
Intelence® (etravirine)	☐	☐		mg		x/j	Date:		Heure:
.....	☐	☐		mg		x/j	Date:		Heure:

ANTAG. CCR5

Celsentri® (maraviroc)	☐	☐		mg		x/j	Date:		Heure:
.....	☐	☐		mg		x/j	Date:		Heure:

INIs

Isentress® (raltegravir)	☐	☐		mg		x/j	Date:		Heure:
.....	☐	☐		mg		x/j	Date:		Heure:

Appendix 2: Questionnaires (SF12 and The Depression Anxiety Stress Scale (DASS) – validated French translation and the Groningen Sleep Quality Score (GSQS, 17))

Cf. pages 15, 16 and 17

SF-12 (Short Form)

Question 1	In general, would you say your health is excellent, very good, good, fair, or poor?	Excellent ...	☐	0
		Very Good ...	☐	0
		Good ...	☐	0
		Fair ...	☐	0
		Poor ...	☐	-2
Question 2	The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? First, moderate activities such as moving a table, pushing a vacuum cleaner, bowling or playing golf. Does your health now limit you a lot, limit you a little, or not limit you at all.	Limited a lot ...	☐	4
		Limited a little ...	☐	2
		Not limited at all ...	☐	0
Question 3	Climbing several flights of stairs. Does your health now limit you a lot, limit you a little, or not limit you at all?	Limited a lot ...	☐	3
		Limited a little ...	☐	1
		Not limited at all ...	☐	0
Question 4	During the past four weeks, have you accomplished less than you would like as a result of your physical health?	No ...	☐	0
		Yes ...	☐	1
Question 5	During the past four weeks, were you limited in the kind of work or other regular activities you do as a result of your physical health?	No ...	☐	0
		Yes ...	☐	2
Question 6	During the past four weeks, have you accomplished less than you would like to as a result of any emotional problems, such as feeling depressed or anxious?	No ...	☐	0
		Yes ...	☐	-7

EDAS

Nom: *Date:*

Veuillez lire chaque énoncé et indiquez lequel correspond le mieux à votre expérience au cours de *la dernière semaine*. Indiquez votre choix en encerclant le chiffre qui y correspond (soit 0,1,2 ou 3). Il n'y a pas de bonne ou de mauvaise réponse. Ne vous attardez pas trop longuement aux énoncés.

L'échelle de notation est la suivante :

0 ne s'applique pas du tout à moi
1 s'applique un peu à moi, ou une partie du temps
2 s'applique beaucoup à moi, ou une bonne partie du temps
3 s'applique entièrement à moi, ou la grande majorité du temps

1	Je me suis aperçu(e) que des choses insignifiantes me troublaient.	0	1	2	3
2	J'ai été conscient(e) d'avoir la bouche sèche.	0	1	2	3
3	J'ai eu l'impression de ne pas pouvoir ressentir d'émotion positive.	0	1	2	3
4	J'ai eu de la difficulté à respirer (par exemple, respirations excessivement rapides, essoufflement sans effort physique).	0	1	2	3
5	J'ai eu de la difficulté à initier de nouvelles activités.	0	1	2	3
6	J'ai eu tendance à réagir de façon exagérée.	0	1	2	3
7	Je me suis senti(e) faible (par exemple, les jambes qui allaient se dérober sous moi).	0	1	2	3
8	J'ai eu de la difficulté à me détendre.	0	1	2	3
9	Je me suis trouvé(e) dans des situations qui me rendaient tellement anxieux(se) que j'ai été très soulagé(e) lorsqu'elles ont pris fin.	0	1	2	3
10	J'ai eu le sentiment de ne rien envisager avec plaisir.	0	1	2	3
11	Je me suis aperçu(e) que j'étais assez facilement contrarié(e).	0	1	2	3
12	J'ai eu l'impression de dépenser beaucoup d'énergie nerveuse.	0	1	2	3
13	Je me suis senti(e) triste et déprimé(e).	0	1	2	3
14	Je me suis aperçu(e) que je devenais impatient(e) lorsque j'étais retardé(e) de quelque façon que ce soit (par exemple dans les ascenseurs, aux feux de circulation, lorsque je devais attendre).	0	1	2	3
15	Je me suis senti(e) étourdi(e).	0	1	2	3
16	J'ai eu l'impression d'avoir perdu goût à presque tout.	0	1	2	3
17	J'ai eu le sentiment de ne pas valoir grand chose comme personne.	0	1	2	3
18	J'ai eu l'impression d'être assez susceptible.	0	1	2	3
19	J'ai transpiré de façon perceptible (par exemple, les mains moites) en l'absence de températures élevées ou d'effort physique.	0	1	2	3
20	J'ai eu peur sans bonne raison.	0	1	2	3
21	J'ai eu le sentiment que la vie n'en valait pas la peine.	0	1	2	3

The Groningen Sleep Quality Score (GSQS, 17)

1. I had a deep sleep last night

2. I feel that I slept poorly last night

3. It took me more than half an hour to fall asleep last night

4. I woke up several times last night

5. I felt tired after waking up this morning

6. I feel that I didn't get enough sleep last night

7. I got up in the middle of the night

8. I felt rested after waking up this morning

9. I feel that I only had a couple of hours' sleep last night

10. I feel that I slept well last night

11. I didn't sleep a wink last night

12. I didn't have trouble falling asleep last night

13. After I woke up last night, I had trouble falling asleep again

14. I tossed and turned all night last night

15. I didn't get more than 5 hours' sleep last night

All items are scored true / false

The first question does not count for the total score

One point if answer is 'true': questions 2, 3, 4, 5, 6, 7, 9, 11, 13, 14, 15

One point if answer is 'false': questions 8, 10, 12

Maximum score 14 points, indicating poor sleep the night before

Table S1: Demographic and genetic characteristics of the study population

Characteristic	**Value**	**% study population**
Sex (No.)		
Men	124	73
Women	45	27
Age (y)		
Median (Range)	47 (30-73)	-
Body weight (kg)		
Median (Range)	77.5 (44-101)	-
Height (cm)		
Median (Range)	179 (153-193)	-
Ethnicity (No.)		
Caucasians	142	83
Africans	16	10
Hispanics	6	4
Asians	5	3
PIs (No.)		
Ritonavir	20	13
Saquinavir	4	3
Lopinavir	15	9
Atazanavir	18	11
NRTIs (No.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (No.)		
Enfuvirtide	4	2
Efavirenz (No.)		
200 mg	1	<1
300 mg	1	<1
400 mg	3	2
600 mg	162	96
700 mg	1	<1
800 mg	1	<1
***CYP2B6* genotype (No.)**		
Hom Ref	77	44
Het LOF	53	33
Hom LOF	23	14
Het GOF	16	9

NRTIs: nucleoside reverse transcriptase inhibitors. PIs: Protease inhibitors. Hom: homozygous. Het: heterozygous. Ref: reference allele. LOF: loss of function allele. GOF: gain of function allele.

Table S2: Primers and Probes for genotyping.

Allele	fSNP	Meth.	Primers	Probe	Size	T°	Ref
CYP2A6							
**1H, *1J*	g.-745A>G	Seq.	F: 5'- CCCTCGAATGTGATCTTCTC –3'; R: 5'- CAGCGGGTTCTCCCAGAAAG –3'	-	582 bp	60°C	This study
**9, *13, *15*	g.-48T>G	Taq.	Assay on demand from Applied Biosystems: C__30634332_10		-	60°C	AB
**2*	g.1799T>A	Taq.	Assay on demand from Applied Biosystems: C__27861808_60		-	60°C	AB
**17*	g.5065G>A	Taq.	Assay on demand from Applied Biosystems: C__34816076_20		-	60°C	AB
**7*, **10*, **19*	g.6558T>C	Seq.	F: 5'-TGCAAGTGTACCTGGCAGGAAA-3'; R: 5'- CGCATCTTCCCCCCATTCTTATA –3'	-	654 bp	62°C	[1]
**5*	g.6582G>T						
**12*	GC	Seq.	F : 5'- GGGGGTGAAGGATCCCAGTACT-3'; R : 5'- GTCCCCTGCTCACCGCCA-3'	-	1474 bp	65°C	[1]
**4/ *1X2*	CNV	qPCR	Assay on demand from Applied Biosystems: HS0001002_cn		-	60°C	AB
β-Globin							
**1*	CNV	qPCR	F : 5'-GGCAACCCTAAGGTGAAGGC-3'; R : 5'-GGTGAGCCAGGCCATCACTA-3'	VIC-5'-CATGGCAAGAAAGTGCTCGGTGCCT-3' TAMRA	67 bp	60°C	[2]

SNP: single nucleotide polymorphism. fSNP: functional SNP. Meth.: Methods. Seq.: Sequencing. Taq.: TaqMan allelic discrimination. qPCR: quantitative PCR. AB: Applied Biosystems. GC: gene conversion. CNV: copy number variations. Position numbering of the functional SNPs refers to genomic (g.) DNA (bp1=A of ATG). F: forward. R: reverse

1. Haberl M, Anwald B, Klein K, Weil R, Fuss C, Gepdiremen A*, et al*. Three haplotypes associated with CYP2A6 phenotypes in Caucasians. *Pharmacogenet Genomics* 2005; **15**(9)**:** 609-624.
2. Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G*, et al*. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science* 2005; **307**(5714)**:** 1434-1440.

Table S3: Primers used for *CYP2A6* re-sequencing.

Region	Primers	Size	T°	Ref
Promoter	F: 5'- CCCTCGAATGTGATCTTCTC –3'; R: 5'-CAGCGGGTTCTCCCAGAAAG –3'	582 bp	60°C	This study
Promoter + Exon 1	F: 5'-GGCTGTGTCCCAAGCTAGGCA-3'; R: 5'-GACTCTGGTCCACACTGGTCAAC-3'	428 bp	62°C	[1]
Exon 2	F : 5'-TCCCTGACTGTGAGAACCTGGGT-3'; R : 5'-GATGGGGAGGGAAGACCAGACT-3'	407 bp	62°C	[1]
GC In 2	F : 5'-GGGGGTGAAGGATCCCAGTACT-3'; R : 5'- GTCCCCTGCTCACCGCCA-3'	1474 bp	65°C	[1]
Exon 3	F: 5'-CTCTGACTGAGTTTGCAGCTCTG-3'; R:5'-AACGCGCGCGGGTTCCTCGT-3'	379 bp	62°C	[1]
Exon 4	F: 5'-GCGCTGGGAATTTGGCTCAACAA-3'; R: 5'-GGGGACACTGTCTGGAGGGC-3'	375 bp	62°C	[1]
Exon 5	F: 5'-GCCCCACTGAAATACCTAAACAAC-3'; R: 5'-CTGCCTGCCCCACTCCCAGA-3'	389 bp	62°C	[1]
Exon 6	F: 5'-CCCTCTTTCCACCTTTGGTCTGA-3'; R: 5'-ATCAGTGCAGACATTTTCAATATTTTAATAT-3'	450 bp	62°C	[1]
Exon 7	F: 5'-TGATGTCTGTTCTGTTATGAATGCTCTACT-3'; R: 5'-GACAGGGTCTAGAAAGCTTCTAATGT-3'	423 bp	62°C	[1]
Exon 8	F: 5'-GTCCCCCAAACTCCTGCCTAGA-3'; R: 5'-TACACCGCAGAGAGGGGAGGA-3'	451 bp	62°C	[1]
Exon 9	F: 5'-GGTTCACCATTGTTACATCTCTTATAGAAAGAAAT-3'; R: 5'-TCCTGCCCCCAGTCTTAGCTG-3'	473 bp	62°C	[1]
GC 3' UTR region	F: 5'-TGCAAGTGTACCTGGCAGGAAA-3'; R: 5'-CGCATCTTCCCCCCATTCTTATA-3'	654 bp	62°C	[1]

GC: Gene conversion. In: intron 2. UTR: untranslated region. F: forward. R: reverse

1. Haberl M, Anwald B, Klein K, Weil R, Fuss C, Gepdiremen A*, et al*. Three haplotypes associated with CYP2A6 phenotypes in Caucasians. *Pharmacogenet Genomics* 2005; **15**(9)**:** 609-624.

Table S4: Primers used to amplify and re-sequence *CYP2A6*34*

Region amplified	PCR-Primers	Size	T°
Promoter *CYP2A7->* intron 5 *CYP2A6*	F: 5'–CCTCCGCAACAGAAGACCTC –3'; R: 5'–TTGAATGGGCCTGTGTCATC –3'	4010 bp	65°C
Exon 1 *CYP2A7->* intron 5 *CYP2A6*	F: 5'–TCCCAAGCTAGGTGGCATTC–3'; R: 5'–TTGAATGGGCCTGTGTCATC–3'	3692 bp	65°C
Exon 2 *CYP2A7->* intron 5 *CYP2A6*	F : 5'–GTGAAGGATCCCAGTACTTG–3'; R : 5'–TTGAATGGGCCTGTGTCATC–3'	3176 bp	65°C
Exon 3 *CYP2A7->* intron 5 *CYP2A6*	F : 5'–TCTCTGCGCATCTCTATCTG–3'; R : 5'–TTGAATGGGCCTGTGTCATC–3'	2260 bp	65°C
Exon 4 *CYP2A7->* intron 5 *CYP2A6*	F: 5'–CGCACGGTGAGTAAGGTTCC–3'; R:5'–TTGAATGGGCCTGTGTCATC–3'	1819 bp	65°C

Region sequenced	Sequencing-Primers	Size	T°
Exon 1	F: 5'–TCCCAAGCTAGGTGGCATTC–3'; R: 5'–CCCAGCACCGAGATGTCAAG –3'	552 bp	50°C
Exon 2	F: 5'–GTGAAGGATCCCAGTACTTG–3'; R: 5'–ATGGAGAGGCCACAGTGAAG –3'	407 bp	50°C
Exon 3	F: 5'–TCTCTGCGCATCTCTATCTG–3'; R: 5'–TCAGAGGTCTGAGGAGAATC–3'	639 bp	50°C
Exon 4	F: 5'–CGCACGGTGAGTAAGGTTCC–3'; R: 5'–GGCTTTTGTTCAGGTGCTCAG –3'	1111 bp	50°C
Recombinant region	F: 5'–AGTTGCACCAGAAGCCTGTC–3'; R: 5'–GGCTTTTGTTCAGGTGCTCAG –3'	569 bp	50°C

Exons 5 to 9 were re-sequenced with the primers shown in Supplementary Table S3.

Table S5: Association of CYP2A6 genotype and EFV plasma exposure

	CYP2A6 alleles (n)				Median log_{10} EFV	Range log_{10} EFV	n
	Ref	DOF<<50%	DOF≥50%	LOF	AUC (μg*h/ml)	AUC (μg*h/ml)	
***CYP2B6* het GOF**	2	-	-	-	1.445	1.389-1.654	8
	1	1	-	-	1.590	1.456-1.611	3
	1	-	1	-	1.362	1.248-1.598	3
	1	-	-	1	1.668	-	1
	-	1	1	-	1.596	-	1
***CYP2B6* reference**	2	-	-	-	1.590	1.188-1.919	51
	1	1	-	-	1.680	1.567-2.129	10
	1	-	1	-	1.569	1.306-1.892	9
	1	-	-	1	1.552	1.526-1.578	2
	-	1	1	-	1.790	1.621-1.957	3
	-	-	2	-	1.673	-	1
	-	-	1	1	1.769	-	1
***CYP2B6* het LOF**	2	-	-	-	1.716	1.458-2.164	30
	1	1	-	-	1.629	1.480-1.975	8
	1	-	1	-	1.727	1.590-1.911	8
	1	-	-	1	1.666	1.632-1.783	3
	-	1	1	-	1.839	1.806-2.011	3
	-	-	1	1	1.734	-	1
***CYP2B6* hom LOF**	2	-	-	-	2.177	1.687-2.639	12
	1	1	-	-	2.264	1.850-2.317	5
	1	-	1	-	2.518	2.286-2.635	3
	-	1	1	-	2.482	-	1
	-	-	2	-	2.464	-	1
	-	-	-	2	2.989	-	1

Hom: homozygous. Het: heterozygous. Ref: reference. DOF/LOF: decreased/loss-of-function.

Table S6: Genetic profiles selected for metabolite analysis.

	***CYP2A6* alleles (n)**				**Individuals**
	Reference	**DOF<50%**	DOF≥50%	LOF	**(n)**
***CYP2B6* reference**	2	-	-	-	11
	1	1	-	-	8
	1	-	1	-	3
	-	1	1	-	3
	-	-	2	-	1
	-	-	1	1	1
***CYP2B6* hom LOF**	2	-	-	-	12
	1	1	-	-	5
	1	-	1	-	2
	-	-	2	-	1
	-	-	-	2	1

Hom: homozygous. Het: heterozygous. Ref: reference. DOF/LOF: decreased/loss-of-function.

Protocole de dosage de l'efavirenz et de certains de ces métabolites dans le plasma

Produits chimiques :

- Méthanol (MeOH)
- Acétonitrile (ACN)
- Eau Ultrapure MilliQ (H_2O)
- Acide formique (FA)
- Ammonium formate

Equipements :

- Tubes Eppendorf + portoirs
- Hotte d'aspiration (chapelle)
- Pipettes *Eppendorf* P20, P200 , P1000 et les embouts correspondants
- Vortex
- Agitateur
- Centrifugeuse Hettich universal 16R pour tubes Eppendorf
- Vials en verre pour HPLC (Hewlett Packard)
- Caps pour vials HPLC (Hewlett Packard)
- HPLC MS/MS
- Colonne Waters Atlantis^{TM}-dC18 3µm 2.1x50mm

Normes de sécurité :

- Les manipulations avec du matériel biologique potentiellement infectieux (plasma) se font toujours avec des gants de protection.
- Les manipulations avec les solvants se font sous une hotte d'aspiration avec des gants et si nécessaire (absence de protection vitrée) en portant des lunettes de protection.

Solutions mères :

- Solution mère 1 (SM1) : Efavirenz (EFV) à 1 mg/ml (ou 1000 µg/ml)
- Solution mère 2 (SM2) : 8-hydroxy-Efavirenz (8-OH-EFV) à 1 mg/ml (ou 1000 µg/ml)

Standard interne :

- Efavirenz-d4 (EFV-d4): solution à 100 µg/ml dans MeOH/H2O 50:50
- 8-OH-Efavirenz-d4 (8-OH-EFV-d4): solution à 10 µg/ml dans MeOH/H2O 50:50

Préparation des calibrateurs et contrôles de qualité :

Solutions	Concentration [ng/ml]	Solution utilisée	Volume de Sol. [µl]	Volume de MeOH/H_2O 50:50 [µl]
A	200'000	SM1+SM2	100+100	300
B	100'000	SM1+SM2	50+50	400
C	50'000	SM1+SM2	25+25	450
D	10'000	B	50	450
E	5'000	C	50	450
F	2'500	C	25	475
J	500	E	50	450
G	150'000	SM1+SM2	105+105	490
H	75'000	G	250	250
I	7'500	G	25	475

A 1800 µl de plasma blanc, on ajoute 200 µl de solution = 2 mL de plasma aux concentrations suivantes :

		Concentration [ng/ml]
Calibrateurs	A	20'000
	B	10'000
	C	5'000
	D	1'000
	E	500
	F	250
	J	50
Contrôles	G	15'000
	H	7'500
	I	750

Aliquoter dans des Eppendorfs à raison de 100 µl de plasma spiké (20 x 100 µl).
Stocker à –20°C.
Ne pas thermiser ! (on ne connaît pas la stabilité des métabolites chez les patients…)

Extraction :

- A 100 µl de plasma, ajouter 50 µl solution EFV-d4 + 50 µl de solution 8OH-EFV-d4, puis 600 µl d'ACN.
- Vortexer et laisser reposer.
- Centrifuger à 14'000 rpm à 4°C pendant 10 minutes.
- Prélever 150 µl du surnageant que l'on place dans un vial d'injection, et y ajouter 150 µl d'un mélange Acétate d'ammonium 20mM / MeOH 50:50.
- Fermer les vials et vortexer.

Paramètres de l'analyse HPLC-MS/MS :

Méthode :

`EFV_EFVd4_8OHEFVandmetabolitefinale080229.meth`

Colonne :

Waters Atlantis™-dC18 3µm 2.1x50mm
Volume d'injection = 20 µl.

Phase mobile :

-solvant A : 10mM Ammonium formate dans H_2O à pH=3.5 avec FA
-solvant B : ACN

Détection :

Scan Event 1 : SRM mode

Drug	Transition	CE	Q1 PW	Q3 PW	TL
Efavirenz	314.0 → 244.0	20	0.70	0.70	60
Efavirenz-d4	318.0 → 248.0	27	0.70	0.70	37
OH-Efavirenz	330.0 → 257.9	31	0.70	0.70	97
8-OH-Efavirenz-d4	334.0 → 257.9	35	0.70	0.70	77

Scan Event 2 : SIM mode

Drug	Parent	Q1 PW	Q3 PW	TL
OH-Efavirenz	330.0	0.70	0.70	77
N-Gln-Efavirenz	490.0	0.70	0.70	77
di-OH-Efavirenz	522.0	0.70	0.70	77
Gln-O-Efavirenz	506.0	0.70	0.70	77

Scan Event 3 : Full MS2 mode

Drug	Parent	Product	CE	Q1 PW	Q3 PW
di-OH-Efavirenz	346.0	Scan from 50.0 to 350.0	20	0.70	0.70

Source CID Collision Energie : 10

Gradient :

Time	Solvant A %	Solvant B %	Débit µL/min
0.00	90.0	10.0	300.0
20.00	0.0	100.0	300.0
20.50	0.0	100.0	500.0
23.00	0.0	100.0	300.0
24.00	90.0	10.0	500.0
30.00	90.0	10.0	300.0

Temps de rétention :

Efavirenz	12.00 min
Efavirenz-d4	12.00 min
7-OH-Efavirenz	9.60 min
8-OH-Efavirenz	10.88 min
N-Gln-Efavirenz	7.66 min
8-OH-Efavirenz-d4	10.84 min

In vivo analysis of efavirenz metabolism in individuals with impaired *CYP2A6* function

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Introduction **The antiretroviral drug efavirenz (EFV) is extensively metabolized into three primary metabolites: 8-hydroxy-EFV, 7-hydroxy-EFV and *N*-glucuronide-EFV. There is a wide interindividual variability in EFV plasma exposure, explained to a great extent by cytochrome P450 2B6 (CYP2B6), the main isoenzyme responsible for EFV metabolism and involved in the major metabolic pathway (8-hydroxylation) and to a lesser extent in 7-hydroxylation. When CYP2B6 function is impaired, the relevance of CYP2A6, the main isoenzyme responsible for 7-hydroxylation may increase. We hypothesize that genetic variability in this gene may contribute to the particularly high, unexplained variability in EFV exposure in individuals with limited CYP2B6 function.**

Methods **This study characterized *CYP2A6* variation (14 alleles) in individuals (*N*=169) previously characterized for functional variants in *CYP2B6* (18 alleles). Plasma concentrations of EFV and its primary metabolites (8-hydroxy-EFV, 7-hydroxy-EFV and *N*-glucuronide-EFV) were measured in different genetic backgrounds *in vivo*.**

Results **The accessory metabolic pathway CYP2A6 has a critical role in limiting drug accumulation in individuals characterized as CYP2B6 slow metabolizers.**

Conclusion **Dual CYP2B6 and CYP2A6 slow metabolism occurs at significant frequency in various human populations, leading to extremely high EFV exposure. *Pharmacogenetics and Genomics* 19:300–309 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The antiretroviral drug efavirenz (EFV) is primarily metabolized by the cytochrome P450 2B6 (CYP2B6) [1]. There is a high interindividual variability in plasma drug levels after drug administration at the usual dosage regimen of 600 mg/day [2]. This observation reflects the existence of slow and rapid metabolizer phenotypes associated with genetic variations in *CYP2B6* [3–7]. Although *CYP2B6* alleles explain to a large extent the observed interindividual variability in EFV exposure, there is a remaining variability, in particular among individuals with impaired CYP2B6 function [5]. This unexplained variability suggests the participation of other genetic and environmental influences in EFV elimination.

EFV is extensively metabolized into three primary metabolites; two of them are hydroxylated metabolites [8-hydroxy-EFV (8-OH-EFV) and 7-hydroxy-EFV (7-OH-EFV)] and the third is a glucuroconjugated product [*N*-glucuronide-EFV (*N*-gln-EFV)] (Fig. 1). 8-Hydroxylation is the main metabolic pathway (92%) and is essentially the result of CYP2B6 activity [1]. Recent *in vitro* data suggest that 7-hydroxylation is the second most important pathway (<8%), mainly because of CYP2A6 activity and to a lesser extent CYP2B6 activity [8] (Fig. 1). *N*-gln-EFV has been identified in human plasma and urine although the pathway has not been characterized [9] (Fig. 1). Here we hypothesize that, in individuals with impaired CYP2B6 function, functional polymorphism in *CYP2A6* may be of clinical importance.

CYP2A6 has been shown to have large interindividual and interethnic variability in levels of expression and activity, and this is thought to be largely because of

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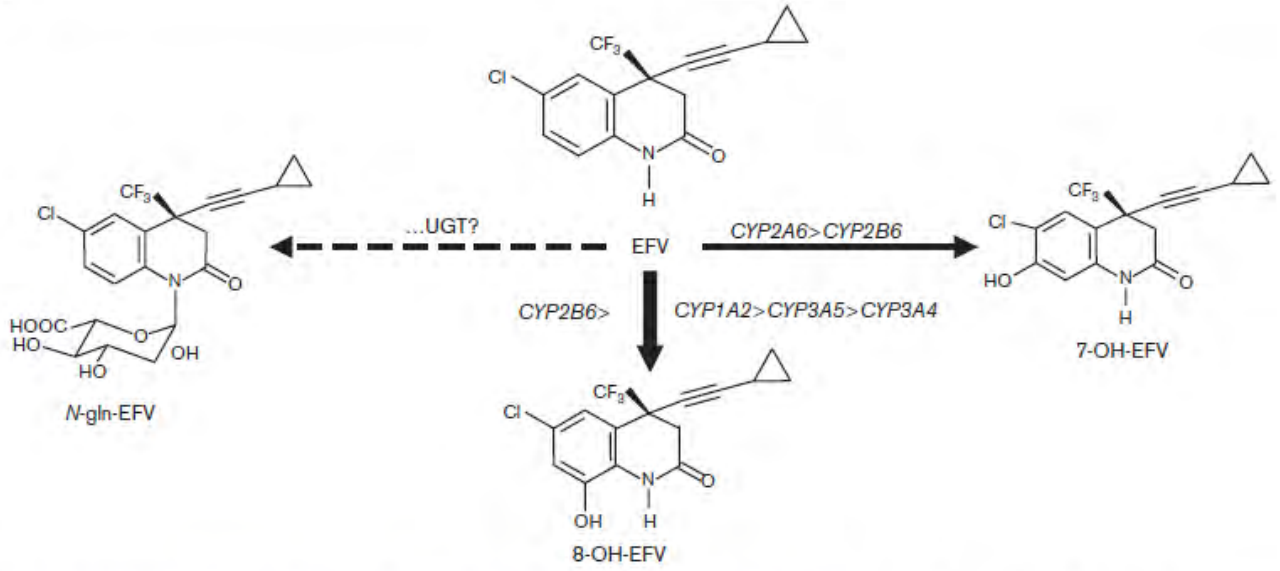
Fig. 1



Efavirenz (EFV) primary metabolic pathways. EFV is extensively metabolized into three primary metabolites: 8-hydroxy-EFV (8-OH-EFV), 7-hydroxy-EFV (7-OH-EFV) and *N*-glucuronide-EFV (*N*-gln-EFV).

genetic polymorphisms [10]. Variation in activity affects the metabolism of CYP2A6 substrates such as nicotine and coumarin.

In this study, we conducted detailed assessment of functional genetic variation in *CYP2A6* in a well-characterized population of HIV-infected individuals receiving EFV, and validated the observed genotypic/phenotypic associations by *in vivo* metabolite profiling.

Materials and methods

Study population

The study group includes 169 individuals extensively characterized for their *CYP2B6* genotype in a previous study [5], and described in Supplementary Table S1. The ethics committees of all participating centres approved the genetics project and participants gave written informed consent for genetic testing.

Population pharmacokinetics

A population pharmacokinetic analysis of EFV was fitted according to our previous study [11] to derive average population parameters and interpatient variability, and to estimate the influence of demographic factors in its elimination. EFV plasma levels were collected as a part of therapeutic drug monitoring protocol according to local treatment guidelines. EFV pharmacokinetics was characterized using a one-compartment model with first order absorption from the gastrointestinal tract and assigned an interindividual variability on oral clearance (CL), assuming a lognormal distribution with mean zero and variance Ω. The influence of demographic covariates (sex, ethnicity, age, body weight, height and other comedications such as antiretroviral drugs and few other medications) was analysed using linear relationships. A proportional error model with a mean of zero and a variance of σ^2 was used to describe intraindividual variability. Individual Bayesian estimates of CL were used to derive individual area under the curves (AUC=dose/CL) that were used for statistical analyses. The population pharmacokinetics analysis was performed with NONMEM (version VI, NM-TRAN version II, Icon Development Solutions, Ellicott City, Maryland, USA) using FOCE INTERACTION to fit the models. Model choice was based on the likelihood ratio test, and goodness-of-fit plots.

CYP2A6 genotyping

Analysis of *CYP2A6* genetic variation included extensive genotyping of known alleles, full resequencing for selected individuals for the identification of new alleles, and study of copy number variation. Genotyping targeted all alleles with known impact on expression or function in Caucasians, as well as the most common decrease-of-function (DOF)/loss-of-function (LOF) alleles found in Africans and Asians (Table 1, and references therein). We used TaqMan allelic discrimination (ABI prism 7000 sds software, Applied Biosystems, Foster City, California, USA) to investigate five alleles (*CYP2A6*2*, *CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*, *CYP2A6*17*). Alleles resulting from gene conversion (*CYP2A6*5*, *CYP2A6*7*, *CYP2A6*10*, *CYP2A6*12*, *CYP2A6*19*) and promoter

Table 1 ***CYP2A6*** **alleles investigated**

Allele	Functional SNP (rs)	Proposed functional consequence	Reference
*CYP2A6*1H, CYP2A6*1J*	g.−745A>G (rs61663607)	DOF (<50% decreased activity *in vitro*)	[12]
*CYP2A6*1×2A−B*	Gene duplication	GOF (increased activity *in vivo*)	[13]
*CYP2A6*2*	g.1799T>A (rs56844942)	LOF (catalytically inactive)	[14]
*CYP2A6*4A−F*	GC with *CYP2A7* in intron 8	LOF (gene deletion)	[15,16]
*CYP2A6*5*	g.6582G>T (rs5031017)	LOF (catalytically inactive)	[17]
*CYP2A6*7, CYP2A6*10, CYP2A6*19*	g.6558T>C (rs5031016)	DOF (≥50% decreased activity *in vitro*)	[18]
*CYP2A6*9, CYP2A6*13, CYP2A6*15*	g.−48T>G (rs28399433)	DOF (≥50% decreased activity *in vivo/in vitro*)	[19]
*CYP2A6*12*	GC with *CYP2A7* in intron 2	DOF (≥50% decreased activity *in vivo/in vitro*)	[20]
*CYP2A6*17*	g.5065G>A (rs28399454)	DOF (≥50% decreased activity *in vivo/in vitro*)	[21]
*CYP2A6*34*	GC with *CYP2A7* in intron 4	LOF (partial gene deletion)	This study

DOF, decrease-of-function allele; GC, gene conversion; GOF, gain-of-function allele; LOF, loss-of-function allele; SNP, single nucleotide polymorphism.

alleles (*CYP2A6*1H*, *CYP2A6*1J*) were genotyped by sequencing. Primers and probes are shown in Supplementary Table S2.

On account of the working hypothesis that *CYP2A6* variation would be clinically relevant among individuals with impaired CYP2B6 metabolism, *CYP2A6* promoter region, and all nine exons and intron–exon boundaries (3775 bp) were fully resequenced in all individuals homozygous for a LOF of *CYP2B6* (n=23). Primers are shown in Supplementary Table S3.

Gene copy number was determined in all individuals, as gene deletions (*CYP2A6*4A–4F*) and gene duplications (*CYP2A6*1×2A–B*) have been described for this gene [13,22–25]. Real-time PCR was performed using the TaqMan ABI prism 7000 sds software (Applied Biosystems). For each analysis, standard curves (seven serial 1:2 dilutions of genomic DNA) were built for a reference gene (*β-globin*, two copies per diploid genome), and for *CYP2A6* in separate wells. *β-globin* primers and probe were published earlier [26] (Supplementary Table S2). *CYP2A6* primers and probe were obtained as assay-on-demand from Applied Biosystems (TaqMan Gene Expression Assays: HS0001002_cn) (Supplementary Table S2). The probe is located in exon 7, which is missing in the gene deletions described in the literature [22–24].

CYP2B6 genotyping

The details of *CYP2B6* genotyping have been presented earlier [5].

Nomenclature

CYP2A6 allele designation was performed on the basis of genotyping of known functional single nucleotide polymorphisms (SNPs), as well as by full resequencing. Novel alleles were designated in concordance with the CYP Allele Nomenclature Committee (*http://www.cypalleles.ki.se*). Position numbering refers to genomic DNA (indicated as g.) according to NT_011109.15 (base pair 1=A of ATG of *CYP2A6*). *CYP2B6* allele designation was established as before [5]. *CYP2A6* investigated alleles were reported as DOF alleles or LOF alleles, according to the known/proposed activity of the encoded protein. The DOF alleles were further broken down into two categories: DOF < 50% (less than 50% decrease in activity or expression) and DOF ≥ 50% (more or equal to 50% decrease in activity or expression) (Table 1).

In vivo metabolite analyses

Plasma concentrations of EFV, 8-OH-EFV, 7-OH-EFV and *N*-gln-EFV were determined by liquid chromatography coupled to triple quadrupole tandem mass spectrometry (LC-MS/MS; Thermo Scientific Corporation, San Jose, California, USA) after protein precipitation with acetonitrile using an adaptation of our previously reported method [27]. 8-OH-EFV-d_4 and 8-OH-EFV were obtained from Toronto Research Chemicals Inc. (North York, Canada). The m/z transition, collision energy (V) and tube lens used for 7-OH-EFV and 8-OH-EFV in the selected reaction monitoring mode were 330.0 → 257.9, 31 and 97, respectively. *N*-gln-EFV was analysed in the selected ion monitoring mode (m/z=490.0).

Chromatographic profiles and LC-MS spectra of EFV metabolites were compared with available data [1,9]. Quantitative analysis of EFV and 8-OH-EFV was performed with matrix-matched calibration samples using the internal standard method (EFV-d_4 and 8-OH-EFV-d_4, respectively), whereas 7-OH-EFV and *N*-gln-EFV levels were expressed in arbitrary units (a.u.) using signal peak areas. The lower limit of quantification is 50 ng/ml for EFV and 8-OH-EFV. Values were adjusted by dose intake for three individuals that were not receiving a 600 mg EFV regimen.

Results

Population pharmacokinetics

Three hundred and ninety-three EFV plasma concentrations provided by 169 HIV-infected individuals (range 0.1–59.4 μg/ml) were analysed. Average CL was 11.3 l/h with an interindividual variability of 65% (coefficients of variability %), volume of distribution (V) was 388 l and the absorption constant (ka) 0.62/h. Body weight was

retained as significant demographic factor, yielding a 70% increase in CL on body weight doubling; it accounted for the effect of height, age and sex and explained 3% of CL variability. Individual AUC values ranged between 15.4 and 436.0 μg × h/ml. Drug interactions were ruled out as significant contributor to EFV pharmacokinetics in this study, as the presence of ritonavir revealed no statistically significant effect on EFV exposure and only a few individuals were coadministered a nonantiretroviral CYP inducer (n=2) or inhibitor (n=4) drug.

CYP2A6 genotyping

Fifty-five (33%) individuals carried one *CYP2A6* DOF or LOF allele and 13 (8%) carried two DOF and/or LOF alleles (Table 2). Analysis of copy number identified three individuals heterozygous for a gene deletion and two individuals heterozygous for gene duplication (Fig. 2a). These two individuals with a *CYP2A6* gene duplication (expected to represent a gain-of-function allele) were included in the group of extensive CYP2A6 metabolizers.

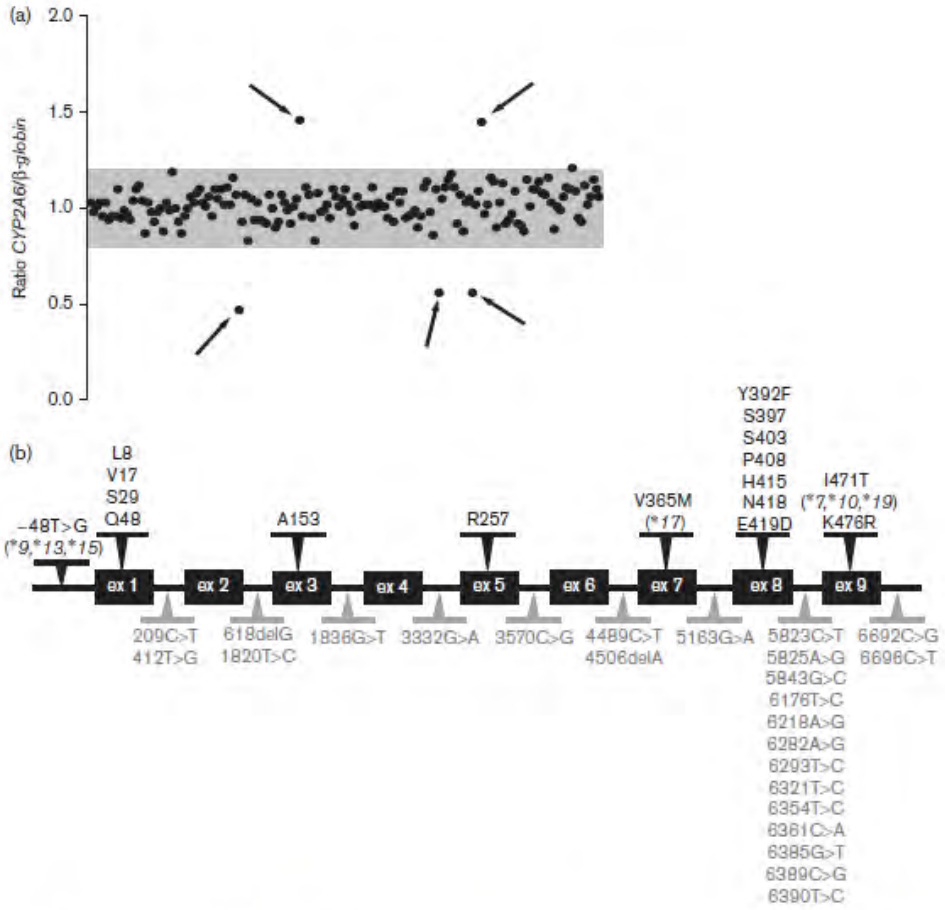
CYP2A6 was fully resequenced in the 23 individuals homozygous for a LOF allele of *CYP2B6* (slow metabolizers). *CYP2A6* resequencing identified one polymorphism in the promoter region, 16 polymorphisms in exons and 25 polymorphisms in exon–intron boundaries. The promoter SNP g.−48T > G (*CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*), modifies the TATA box leading to a decreased expression of the gene [19,28,29]; five exonic SNPs are nonsynonymous, two of which are associated with a DOF (g.5065G > A [V365M], in *CYP2A6*17* [21]; g.6558T > C [I471T], in *CYP2A6*7*, *CYP2A6*10*, *CYP2A6*19* [18]) (Fig. 2b). In addition, resequencing identified an individual that carried a deletion of the first

Table 2 Frequency of *CYP2A6* genotypes in different ethnicities

Region	Allele(s)	SNP	All participants, % (*n*)	Caucasians, % (*n*)	Africans, % (*n*)	Hispanics, % (*n*)	Asians, % (*n*)
5′ UTR	*CYP2A6*1H*, *CYP2A6*1J*	−745A>G					
		AA	79.8 (134)	81.6 (115)	62.5 (10)	100.0 (7)	50.0 (2)
		AG	20.2 (34)	18.4 (26)	37.5 (6)	0.0 (0)	50.0 (2)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
5′ UTR	*CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*	−48T>G					
		TT	83.9 (141)	81.6 (121)	62.5 (10)	85.7 (6)	100.0 (4)
		TG	15.5 (26)	18.4 (20)	31.3 (5)	14.3 (1)	0.0 (0)
		GG	0.6 (1)	0.0 (0)	6.2 (1)	0.0 (0)	0.0 (0)
Exon 3	*CYP2A6*2*	1799T>A					
		TT	96.4 (162)	96.5 (136)	100.0 (16)	85.7 (6)	100.0 (4)
		TA	3.6 (6)	3.5 (5)	0.0 (0)	14.3 (1)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 7	*CYP2A6*17*	5065G>A					
		GG	98.2 (166)	100.0 (141)	81.3 (13)	100.0 (7)	100.0 (5)
		GA	1.8 (3)	0.0 (0)	18.7 (3)	0.0 (0)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	*CYP2A6*7*, *CYP2A6*10*, *CYP2A6*19*	6558T>C					
		TT	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		TC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		CC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	*CYP2A6*5*	6582A>G					
		AA	100.0 (169)	100 (141)	100.0 (16)	100.0 (7)	100.0 (5)
		AG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 2	*CYP2A6*12*	GC with *CYP2A7*					
		No GC	98.2 (165)	98.6 (139)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.8 (3)	1.4 (2)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 4	*CYP2A6*34*	GC with *CYP2A7*					
		No GC	99.4 (168)	100.0 (141)	100.0 (16)	100.0 (7)	80.0 (4)
		Het GC	0.6 (1)	0.0 (0)	0.0 (0)	0.0 (0)	20.0 (1)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8→3′UTR	*CYP2A6*4A–F* (deletion)	GC with *CYP2A7*					
		No GC	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		Het GC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8→3′UTR	*CYP2A6*1 × 2A–B* (duplication)	GC with *CYP2A7*					
		No GC	98.8 (167)	99.3 (140)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.2 (2)	0.7 (1)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)

The individual genotype frequency was calculated based on the number of individuals for whom genotyping for the selected SNPs has been successfully performed (n). SNP position is based on genomic (g.) DNA numbering (base pair 1 = A of ATG).
GC, gene conversion; Het, heterozygous; Hom, homozygous; SNP, single nucleotide polymorphism; UTR, untranslated region.

Fig. 2



CYP2A6 genotyping. (a) Gene copy number determination. Each dot represents one individual ($N=169$). The grey box represents the *CYP2A6/β-globin* ratio interval for individuals with two *CYP2A6* copies (ratio average value=1.02, 95 percentile, 0.87–1.16). Black arrows indicate the individuals heterozygous for a gene deletion (*CYP2A6*4A–F*) (ratio 0.47/0.56/0.56, respectively, from left to right) and individuals heterozygous for a gene duplication (*CYP2A6*1 × 2A–B*) (ratio 1.47/1.45, respectively, from left to right). (b) Single nucleotide polymorphisms (SNPs) identified by resequencing *CYP2A6* in the individuals *CYP2B6* homozygous loss-of-function (LOF) ($n=23$). There is one decrease-of-function (DOF) SNP (−48T>G) in the promoter region (TATA box), 11 synonymous SNPs, three nonsynonymous SNPs and two nonsynonymous DOF SNPs (V365M, I471T) in the exons, and 25 SNPs in the intron–exon boundaries. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. ex, exon.

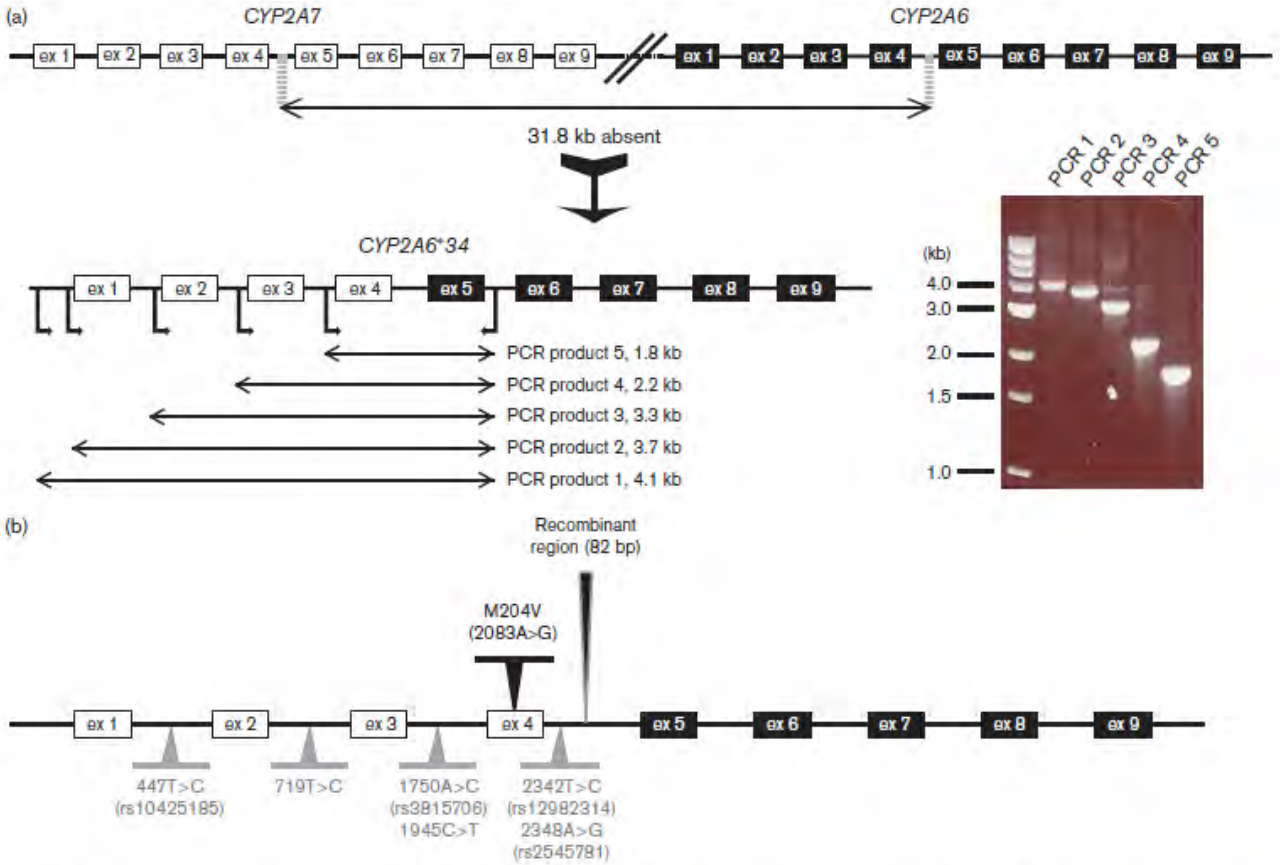
four exons of *CYP2A6*. This new partial gene deletion could not have been identified by gene copy number determination using a probe in exon 7. The new allele results from an unequal crossover in intron 4 between *CYP2A7* and *CYP2A6* (Fig. 3a and b). Primers used for the identification of the new allele are shown in Supplementary Table S4. Further analyses concluded that the patient carried both a known deletion (allele **4A*) on one chromosome, as well as the new allele (*CYP2A6*34*, GenBank accession: EU814898) on the second chromosome. The new allele has 10 amino acid substitutions characterizing the DOF allele *CYP2A6*12* [20] and substitutions R128L and S131A present in the LOF allele *CYP2A6*26* [24], as well as additional nonsynonymous variants. Thus, this partial gene deletion was considered a LOF allele.

Association of *CYP2A6* genotype and efavirenz plasma exposure

Without consideration for CYP2B6 function, *CYP2A6* allele functions seem to have a limited effect on EFV exposure (Fig. 4a). Individuals with reference alleles had a median log10 EFV AUC of 1.67 μg × h/ml, individuals carrying one DOF or LOF allele had a median log10 EFV AUC ranging from 1.64 to 1.68 μg × h/ml ($P=0.29$ vs. reference alleles), whereas individuals carrying two DOF and/or LOF alleles had a median log10 EFV AUC ranging from 1.75 to 2.99 μg × h/ml ($P=0.002$ vs. reference alleles).

On account of the working hypothesis that *CYP2A6* genotype would be clinically relevant among individuals with impaired CYP2B6 metabolism, data were stratified

Fig. 3



*CYP2A6*34* identification and characterization. (a) Identification of a new hybrid allele (*CYP2A6*34*). PCRs were run with forward primers specific to *CYP2A7* and reverse primer specific to *CYP2A6*. (b) Single nucleotide polymorphisms (SNPs) identified by resequencing all exons and intron–exon boundaries of *CYP2A6*34*. White boxes represent exons of *CYP2A7* origin; black boxes represent exons of *CYP2A6* origin. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. ex, exon.

according to the number of *CYP2B6* functional alleles. As this stratification reduced the number of individuals per category, all *CYP2A6* DOF and LOF alleles were referred here as DOF/LOF alleles in order to increase the power (the raw results are shown in Supplementary Table S5). The contribution of *CYP2A6* alleles was particularly relevant among individuals CYP2B6 slow metabolizers. Here, individuals with common *CYP2A6* alleles presented lower median log10 EFV AUC values of 2.18 μg × h/ml, compared with individuals carrying one DOF/LOF allele (2.28 μg × h/ml), and individuals homozygous for a DOF/LOF allele (2.48 μg × h/ml), $P=0.06$ (Fig. 4b).

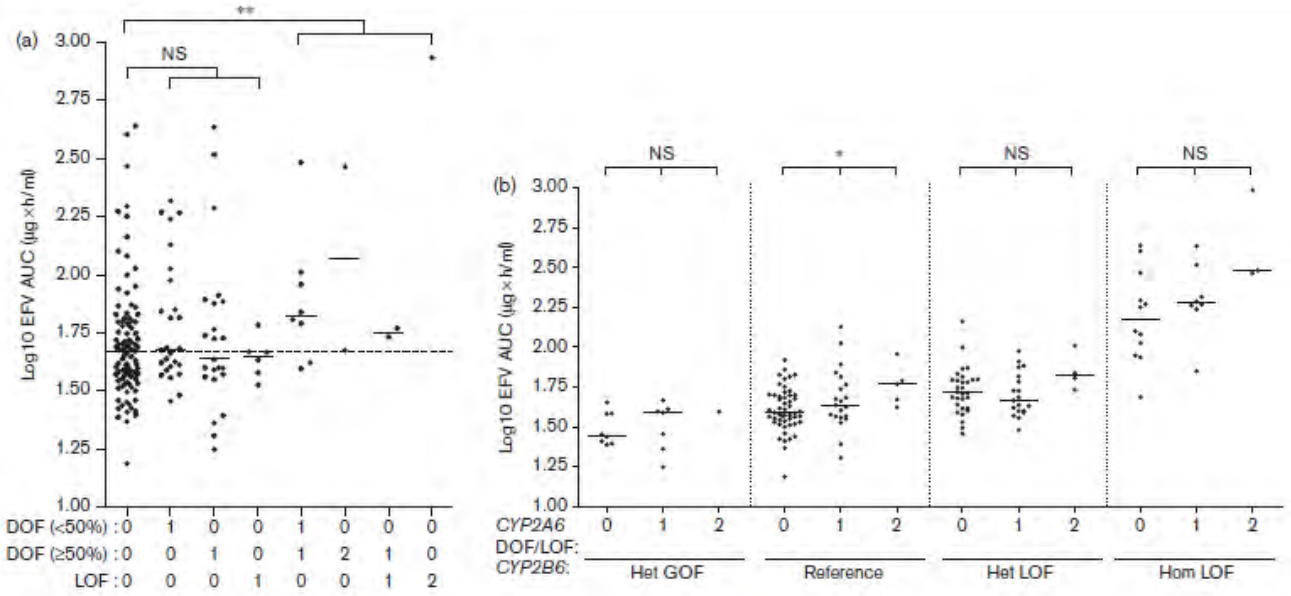
In vivo metabolite analyses

To support the genotyping results, we assessed the concentration of 8-OH-EFV, 7-OH-EFV and *N*-gln-EFV in different genetic backgrounds *in vivo*. For this analysis, we identified individuals ($n=48$) representative of the various genetic profiles (Supplementary Table S6). Chromatogram profiles of individuals with three different genetic backgrounds are shown in Fig. 5a–c.

CYP2B6 slow metabolizers, when compared with CYP2B6 extensive metabolizers, presented an 11% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 6% decrease in the median log10 7-OH-EFV (a.u.) – consistent with a role of CYP2B6 in this pathway (Fig. 1) – and a 35% increase in the median log10 *N*-gln-EFV (a.u.). The limited decrease in 8-OH-EFV and 7-OH-EFV concentrations was the result of the significant increase of the parent compound. When results were expressed as ratios, we observed a 67% decrease in the median log10 8-OH-EFV/EFV ratio, a 33% decrease in the median log10 7-OH-EFV/EFV ratio and a 5% decrease in the *N*-gln-EFV/EFV ratio.

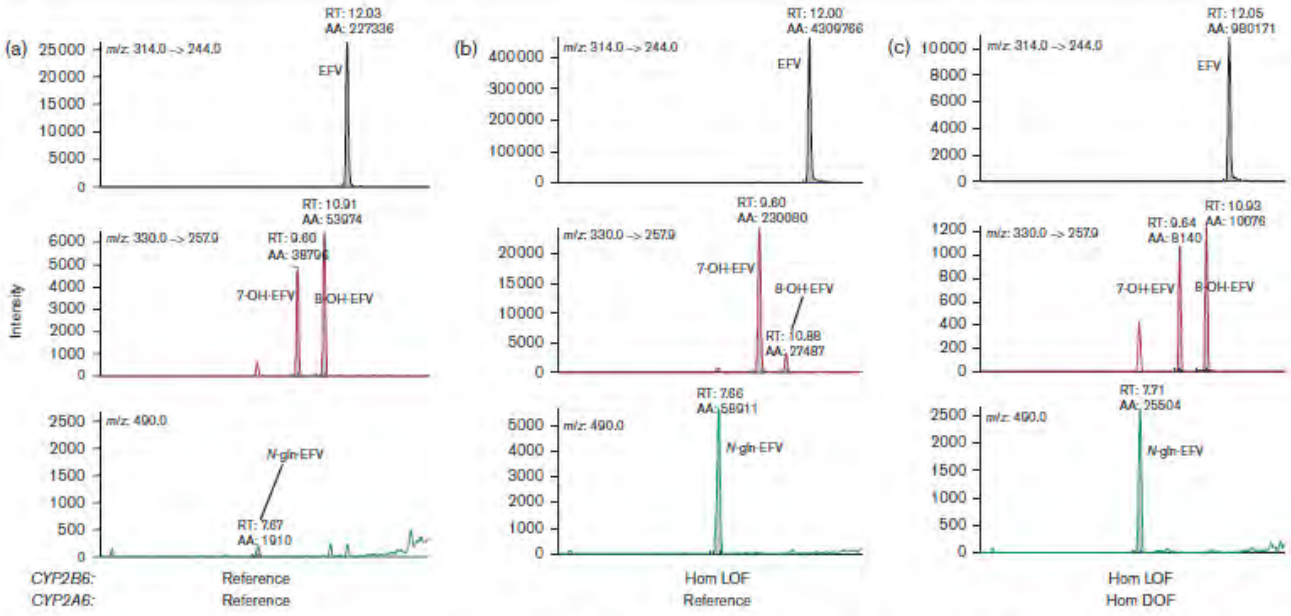
Carriers of one and two *CYP2A6* DOF/LOF alleles, when compared with CYP2A6 extensive metabolizers, were

Fig. 4



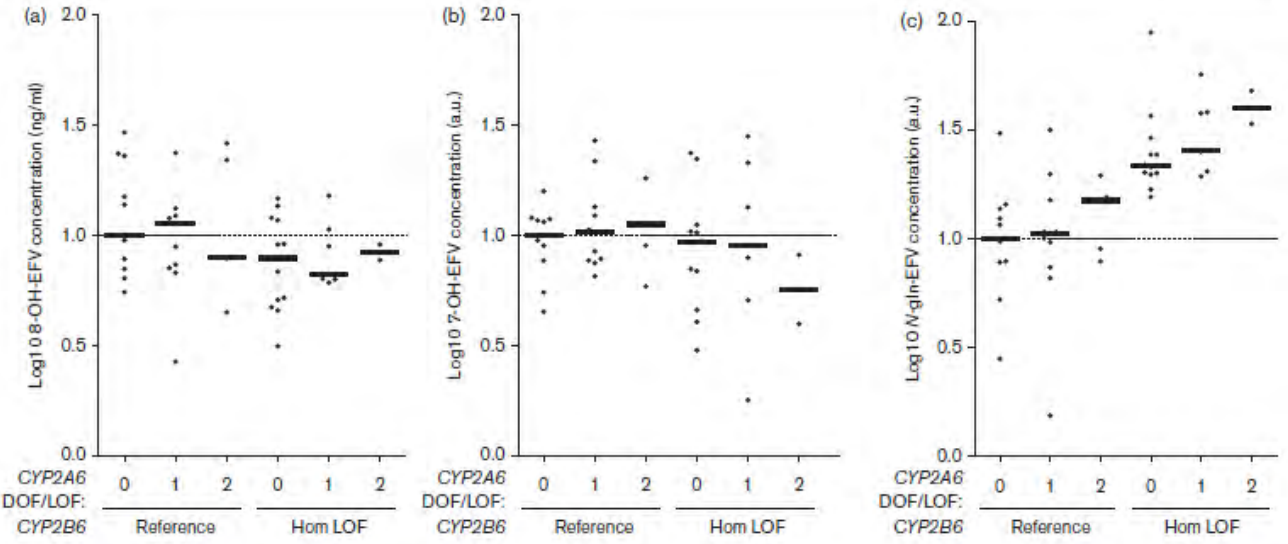
Association of *CYP2A6* genotype and efavirenz (EFV) plasma exposure. (a) Individuals were classified according to their number of *CYP2A6* decrease-of-function (DOF) <50%, DOF ≥ 50% and/or loss-of-function (LOF) alleles. (b) Data were stratified according to *CYP2B6* genotype and according to the number (0, 1 or 2) of *CYP2A6* DOF and/or LOF alleles, all referred to here as DOF/LOF because of the small number of individuals in each category. AUC, area under the curve; GOF, gain-of-function; Het, heterozygous; Hom, homozygous; NS, non significant; Reference, reference alleles. $*P<0.05$, $**P<0.005$.

Fig. 5



Efavirenz (EFV) metabolite profiling. Plasma concentrations of EFV, 7-hydroxy-EFV (7-OH-EFV), 8-hydroxy-EFV (8-OH-EFV) and *N*-glucuronide-EFV (*N*-gln-EFV) were determined by liquid chromatography coupled with triple quadrupole tandem mass spectrometry. Shown are chromatographic profiles of plasma samples from three individuals with representative genotypes. (a) Reference alleles for *CYP2B6* and *CYP2A6*. (b) Homozygous (Hom) loss-of-function (LOF) *CYP2B6* (**6/*6*), and reference alleles for *CYP2A6*. (c) Hom LOF *CYP2B6* (**6/*18*) and Hom decrease-of-function (DOF) *CYP2A6* (**9/*9*). Note that the scale varies for the different profiles. Reference, reference alleles; RT, retention time.

Fig. 6



EFV metabolite concentration in individuals with different *CYP2B6* and *CYP2A6* genetic profiles. EFV metabolite concentrations were stratified according to *CYP2B6* genotype (Reference or Hom LOF) and according to the number (0, 1 or 2) of *CYP2A6* DOF/LOF alleles. (a) 8-OH-EFV concentration (ng/ml). (b) 7-OH-EFV concentration (a.u.). (c) N-gln-EFV concentration (a.u.). The median log10 value for CYP2B6 and CYP2A6 extensive metabolizers is normalized to 1. Reference, reference alleles; DOF/LOF, decrease/loss-of-function; Hom, homozygous.

associated with a 1 and 6% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 1% increase and a 2% decrease in the median log10 7-OH-EFV (a.u.) and a 0 and 3% decrease in the median log10 *N*-gln-EFV (a.u.), respectively.

Data were then stratified according to the number of *CYP2B6* functional alleles. The contribution of *CYP2A6* alleles was more relevant among *CYP2B6* slow metabolizers. In this group, carriers of one and two *CYP2A6* DOF/LOF alleles, when compared with *CYP2A6* extensive metabolizers, presented an 8% decrease and a 3% increase in the median log10 8-OH-EFV concentration (ng/ml), a 2 and 22% decrease in median log10 7-OH-EFV (a.u.) and a 6 and 20% increase in the median log10 *N*-gln-EFV (a.u.), respectively (Fig. 6a–c). These results are consistent with the role of CYP2A6 as an alternative pathway in EFV metabolism, and the role of *N*-glucuronidation in the setting of multiple DOF/LOF in main and accessory hydroxylating pathways.

Discussion

Detailed genetic analysis of EFV metabolic pathways allows a better understanding of the interindividual variability in EFV plasma exposure. It highlights the critical role of the accessory metabolic pathways in limiting drug accumulation in individuals characterized as CYP2B6 slow metabolizers.

We performed a comprehensive assessment of *CYP2A6* genetic variations because of the role of this isoenzyme in the *in vitro* formation of 7-OH-EFV [8]. Analysis included a large number of functional alleles associated with impaired CYP2A6 function, and the assessment of gene copy number. In addition, we identified a new functional genetic variant in this locus through gene resequencing that results from the recombination of *CYP2A7* and *CYP2A6*. Overall, 70 of 169 study participants carried one or more DOF/LOF alleles or copy number variants. The pharmacokinetic data confirmed to a large extent the reported or predicted functional effect of these variants.

Analysis of the primary EFV metabolites *in vivo* showed results consistent with the predicted balance between the main and accessory hydroxylating pathways. LOF *CYP2B6* alleles were associated with a decrease in EFV hydroxy metabolites as previously reported by *in vitro* studies [8], whereas increased levels of *N*-gln-EFV were observed indicating the redirection of the metabolism through this accessory pathway. Among CYP2B6 extensive metabolizers, *CYP2A6* DOF/LOF alleles did not modify EFV 7-hydroxylation because this pathway is still supported by CYP2B6 in the absence of CYP2A6 activity [8]. However, in the presence of impaired CYP2B6 function, *CYP2A6* DOF/LOF alleles were associated with a further decrease in 7-OH-EFV – consistent with the hypothesis that this alternative pathway becomes increasingly relevant in the context of impaired CYP2B6 function – and with a marked increase in *N*-gln-EFV. Analysis of *N*-gln-EFV is particularly reliable because it is

not further metabolized in humans, and the concentration is not dependent on other isoenzyme genetic profiles [9].

This study is limited by the incomplete understanding of functional variations in other genes involved in EFV metabolism. We could not investigate variations of the gene(s) involved in *N*-glucuronidation because of lack of information on the UDP glucuronosyl transferase isoenzyme responsible of this step, and the large number of members in the family. Although CYP1A2 was reported to play a role in EFV metabolism, leading to 8-OH-EFV [1], report on functional alleles has been mostly limited to Asians (*http://www.cypalleles.ki.se* [30]). There is a paucity of functional alleles in *CYP3A4* despite extensive investigation by many groups over the years [31], and the low frequency of functional polymorphisms found in the coding regions cannot account for the variation observed [32]. In addition, a number of factors may affect CYP3A expression, tissue-specific splicing, variable control of gene transcription by endogenous and exogenous molecules and genetic variations in proteins that regulate CYP3A expression through nuclear hormone receptors [33].

This study indicates that the presence of multiple LOF alleles at both the main (CYP2B6) and accessory (CYP2A6) hydroxylation pathways results in extremely high EFV exposure. The clinical relevance of dual CYP2B6 and CYP2A6 slow metabolism is determined by the frequency of DOF/LOF alleles in various human populations. Indeed, the LOF allele *CYP2B6*6* can reach very high frequencies in the population (up to 26% in Caucasians, 47% in Africans and 18% in Asians) [5,34]. Similarly, the prevalence of *CYP2A6* alleles lacking or showing reduced enzymatic activity is elevated in the various populations (9% in Caucasians, 22% in Africans and up to 50% in Asians) ([35] and this study). Therefore, dual CYP2B6 and CYP2A6 slow metabolism will occur at significant frequency in various populations, leading to extremely high EFV exposure in a relevant proportion of individuals. A formal population pharmacokinetic–pharmacogenetic analysis of the data is presented in a separate publication [36].

The approach of identifying functional alleles in multiple metabolic pathways, in combination with metabolite assessment *in vivo* can be of general interest for the validation of *in vitro* studies for other drugs, and to complement pharmacological analysis during drug development.

Supplementary data

Supplementary data are available at *The Pharmacogenetics and Genomics Journal Online* (*www.pharmacogeneticsandgenomics.com*).

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Pharmacogenetics-Based Population Pharmacokinetic Analysis of Efavirenz in HIV-1-Infected Individuals

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Besides CYP2B6, other polymorphic enzymes contribute to efavirenz (EFV) interindividual variability. This study was aimed at quantifying the impact of multiple alleles on EFV disposition. Plasma samples from 169 human immunodeficiency virus (HIV) patients characterized for *CYP2B6*, *CYP2A6*, and *CYP3A4/5* allelic diversity were used to build up a population pharmacokinetic model using NONMEM (non-linear mixed effects modeling), the aim being to seek a general approach combining genetic and demographic covariates. Average clearance (CL) was 11.3 l/h with a 65% interindividual variability that was explained largely by *CYP2B6* genetic variation (31%). *CYP2A6* and *CYP3A4* had a prominent influence on CL, mostly when *CYP2B6* was impaired. Pharmacogenetics fully accounted for ethnicity, leaving body weight as the only significant demographic factor influencing CL. Square roots of the numbers of functional alleles best described the influence of each gene, without interaction. Functional genetic variations in both principal and accessory metabolic pathways demonstrate a joint impact on EFV disposition. Therefore, dosage adjustment in accordance with the type of polymorphism (*CYP2B6*, *CYP2A6*, or *CYP3A4*) is required in order to maintain EFV within the therapeutic target levels.

Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, is widely used in combination with nucleoside inhibitors as first-line treatment of type I human immunodeficiency virus (HIV-1) infection. It is generally prescribed at a fixed dosage of 600 mg daily, despite the presence of a marked interindividual variability in tendency to produce elevated plasma drug concentration levels[1–3] that have been shown to be associated with central nervous system toxicity.[4–6]

EFV is metabolized primarily by *CYP2B6* and, to a lesser extent, by accessory pathways involving *CYP2A6*, *CYP3A4/3A5*, and uridine-glucuronyl-transferases.[7–9] Several studies have shown that *CYP2B6* is highly polymorphic and that genetic variations play an important part in EFV plasma concentration variability.[5,10–15] Genetic polymorphisms of *CYP3A4/3A5* have also been associated with higher EFV exposure,[6] but the influence of the *CYP2A6* polymorphism on EFV pharmacokinetics has not yet been characterized. Considering the increasing number of allelic variants that are being described and the resulting complexity of allele combinations that could influence EFV elimination, we conducted a population pharmacokinetic analysis in HIV-1-infected individuals fully characterized for *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. Our main areas of focus were: (i) to assess the relative contributions of multiple functional alleles involved in EFV elimination along with other demographic or environmental factors, (ii) to characterize the nature of the relationship between individual allelic constitution and EFV disposition, and (iii) to explore models for gene–gene interactions that could lead to a better understanding of the interrelationships of specific enzymes involved in EFV elimination.

RESULTS

In total, 393 plasma samples were collected from 169 individuals. Concentration measurements ranged between 100 and 59,400 ng/ml. A one-compartment model with first-order absorption from the gastrointestinal tract fitted the data

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appropriately. Average clearance (CL) was 11.3 l/h with an interindividual variability of 65%, the volume of distribution (V) was 388 l, and the absorption constant (k_a) was 0.62 h^{-1}. The assignment of interindividual variability on either V or k_a did not improve the fit (change in objective function (ΔOF) = 0.0).

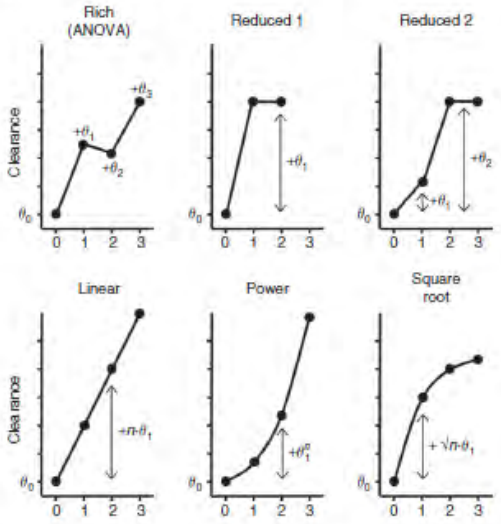

Figure 1 Various parameter models were tested to describe the level of oral clearance (*Y*-axis) as a function of the number of functional alleles of an enzyme (*X*-axis): 0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF (a specific parameter for Het-GOF, is required only with *CYP2B6*). ANOVA, analysis of variance; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

Demographic analyses

Body weight and black ethnicity influenced CL, as did gender, age and, to a lesser extent, height. Co-medications were shown to have no significant influence on EFV pharmacokinetics. A multivariable combination of demographic factors revealed that body weight accounted for the effect of height, age, and gender while explaining 3% of CL variability, and it was the only demographic factor influencing CL besides black ethnicity, which remained statistically significant beyond body weight and reduced the variability in CL by another 3%.

Univariate genotype analyses

The influence of *CYP2B6*, *CYP2A6*, *CYP3A4/AA5* functional alleles on EFV CL was first tested in single-gene analyses, in which the allelic variants (Hom-LOF, Het-LOF, and Hom-Ref plus Het-GOF for *CYP2B6*) were entered into the model as covariates that partitioned individuals based on their genetic constitution.

Genetic variation of *CYP2B6* had by far the most salient impact on CL. Several competing models were tested, as depicted in Figure 1. The richest possible model, which assigned a separate fixed effect to each of the *CYP2B6* allelic variants (Eqs. 1/1a), markedly improved the fit and explained 31% of the 65% interindividual variability on CL. The average CL was 2.8 l/h in the Hom-LOF group and 10.8, 13.3, and 18.8 l/h in individuals carrying Het-LOF, Hom-Ref, and Het-GOF alleles, respectively. A series of reduced models showed CL to be statistically different among all *CYP2B6* groups (ΔOF = +9 and ΔOF = +10 for model reduced 1 and 2). Competing simplified models were tried to

Table 1 Functional alleles evaluated in the study and genotype-based activity score classification

Functional alleles					
Functional consequence		**CYP2B6 alleles**	**CYP2A6 alleles**	**CYP3A4 alleles**	**CYP3A5 alleles**
Loss of function (LOF)		*11, *15, *28	*2, *4		*3, *6, *7, *10, *11
Diminished function (DOF)		*6, *18, *27, *29	*1H, *1J, *5, *7, *9, *10, *12, *13, *15, *17, *19, *34	rs4646437 *1B	—
>25%			*5, *7, *9, *10, *12, *13, *15, *17, *19, *34	—	—
<25%			*1H, *1J	—	—
Reference		*1, *2, *3, *5, *17	*1	*1	*1
Gain of function (GOF)		*4, *22	*1X2	—	—
Genotypes and activity score classification					
Score A	**Alleles (allele 1/allele 2)**	**Score B**	**Alleles (allele 1/allele 2)**	**Score C***	**Alleles (allele 1/allele 2)**
0	LOF/LOF LOF/DOF DOF/DOF	0	LOF/LOF	0	LOF/LOF
1	Ref/LOF Ref/DOF	0.25	LOF/DOF	0.25	LOF/DOF
2	Ref/Ref	0.5	Ref/LOF DOF/DOF	0.5	Ref/LOF
3	Ref/GOF	1	Ref/LOF	0.75	DOF/DOF
		1.5	Ref/DOF	1	Ref/DOF >25%
		2	Ref/Ref	1.5	Ref/DOF <25%
				2	Ref/Ref

DOF, diminished function; GOF, gain of function; LOF, loss of function; Ref, reference allele.

*Only for CYP2A6 alleles.

estimate CL as a function of the number of functional alleles, as defined by the activity score A (Table 1) and were compared with the richest model (Eqs. 1/1a); the use of a linear model (Eq. 2) or a power function model (Eq. 3) did not fit the data appropriately (ΔOF = +18.5 and +58.8, respectively). As interim explorations showed CL to be modestly reduced by ~25% in Het-LOF carriers but cut down by 75% in Hom-LOF individuals, a square root function model achieved the best fit using either an additive (Eq. 4) or a proportional model (ΔOF = −167.7). The recourse to alternative activity scores B for *CYP2B6*, allowing for the distinction between loss and decrease of function alleles, did not better characterize the genotype–phenotype relationships using Eq. 2, 3, or 4 (ΔOF >+8.2).

The assignment of *CYP2A6* allelic variants on CL using the richest model (Eqs. 1/1a) improved the fits (ΔOF = −7.9) and decreased by 1% the overall variability on CL. The average CL was 7.0, 10.8, and 12.1 l/h in Hom-LOF, Het-LOF, and Hom-Ref individuals, respectively. The difference in CL between Hom-Ref and Het-LOF individuals was not significant (ΔOF = −1.0). The description of the relationship between CL and the functional score, using either linear (Eq. 2) or power (Eq. 3) models, did not fit the data adequately when compared with the richest model (ΔOF >+6.8), whereas it was again best characterized using a square root function (Eq. 4) that provided a fit almost identical to that of the rich model (ΔOF = −7.8). Here too, the model integrating partial activity levels (scores B/C) did not improve data description (ΔOF = −0.4 for score B and −0.1 for score C).

The impact of *CYP3A4* on EFV CL was tested using two alleles associated with changes in function, *CYP3A4*1B* and *CYP3A4_* rs4646437. The rich model (Eqs. 1/1a) showed that both alleles influenced CL to a significant extent (ΔOF = −25.4 for *CYP3A4_* rs4646437, −10.4 for *CYP3A4*1B*). CL in Hom-LOF, Het-LOF, and Hom-Ref carriers were, respectively, 5.1, 10.3, and 11.9 l/h for *CYP3A4*1B* and 3.7, 10.7, and 12.3 l/h for *CYP3A4_* rs4646437 alleles. After inclusion of *CYP3A4*1B* and *CYP3A4_* rs4646437 alleles in the model, interindividual variability in CL dropped from 65 to 62 and 59%, respectively. The differences in CL between Hom-Ref and Het-LOF individuals were not significant, both for **1B* and for rs4646437 (ΔOF = −1.4 for *CYP3A4_* rs4646437 and −0.9 for *CYP3A4*1B*). When compared with the richest model, linear and power function models (Eqs. 2/3) did not fit the data adequately (ΔOF >+8.0); square root models (Eq. 4) described the data best (ΔOF = −10.3 for *CYP3A4*1B* and −24.8 for *CYP3A4_* rs4646437).

The influence of *CYP3A5* functional alleles on CL was shown to be small but significant, using the rich model (Eqs. 1/1a, ΔOF = −8.1), with a residual 64% interindividual variability. The difference in CL between individuals with Het-LOF allele and those with Hom-Ref allele was not significant (ΔOF = −0.0). None of the above models (Eqs. 2/3) could characterize the relationship between CL and *CYP3A5* allele variants better than the square root model could (Eq. 4, ΔOF = −8.2).

The effect of black ethnicity remained a statistically influencing covariate on CL in addition to genetic variation, causing an additional 25–40% decrease in CL when associated with functional alleles (ΔOF compared to *CYP2B6* = −8.1, *CYP2A6* = −9.9, *CYP3A4*1B* = −4.0, and *CYP3A5* = −7.8), except for *CYP3A4_* rs4646437 (ΔOF = −0.4), which was present in most of the black individuals, thus limiting the power to detect any association.

Gene–gene interaction analyses

The joint influence of functional alleles on EFV CL was first tested through the conjunction of *CYP2B6* with each of the other *CYP* alleles in dual-gene models, to finally build up the model including all genetic variables having influence on CL. The richest model (Eq. 5) characterizing the joint influence of *CYP2B6* and *CYP2A6*, using a fixed effect parameter for each allelic combination, suggested an additional contribution of *CYP2A6* in EFV elimination (ΔOF = −20 as compared to the final model for *CYP2B6*, Eq. 4). Competitive models were developed based on a functional score A (Table 1) using square root function models. The models evaluating the contribution of *CYP2A6* variation, whether on each *CYP2B6* genotypic group separately (Eq. 6) or through the use of a single parameter estimate across all *CYP2B6* allelic variants (Eq. 7), fitted the data with similar adequacy (ΔOF >−16), resulting in an absolute increase in CL of 1.2 and 1.7 l/h in *CYP2A6* Het-LOF and Hom-Ref individuals, respectively, as compared to Hom-LOF carriers. The contribution of *CYP2A6* functional alleles was more prominent in *CYP2B6* Hom-LOF carriers, in whom the relative change in CL was estimated to be 44% per active allele as compared to an 11% change in Hom-Ref individuals. The joint gene influence could be further described by simply adding *CYP2B6* and *CYP2A6* square root functions (Eq. 8, ΔOF = −12). This model was not considered statistically different from previous models (Eqs. 6/7), considering the reduced degrees of freedom and the loss of fit from the richest possible model (Eq. 5, ΔOF = +8). The introduction of a single interaction factor (Eq. 9) to evaluate some hyper- or hypo-additive trend did not produce any significant change (ΔOF = +3).

The same paradigm was successfully applied to characterize the other gene–gene interactions. The rich model (Eq. 5) characterizing the joint influence of *CYP2B6* with the *CYP3A4*1B* or *CYP3A4* rs4646437 alleles suggested an additive effect of both genes on EFV CL (ΔOF = −14 for **1B*, −25 for rs4646437). Reduced models integrating square root functions of *CYP3A4* activity scores on *CYP2B6* stratified by allelic variation (Eq. 6) described the data appropriately (ΔOF = −13 for **1B*, −18 for rs_4646437), and no deterioration of the fit was observed when allowing a single parameter estimate (Eq. 7) for the effect of *CYP3A4* across all *CYP2B6* allelic variants (ΔOF = +1.0 for **1B*, 0.0 for rs_4646437 as compared to Eq. 6). The additive contribution of *CYP3A4* on CL was 1.1 and 1.5 l/h in **1B* Het-LOF and Hom-Ref carriers, respectively, and 1.4 and 1.9 l/h in rs_4646437 Het-LOF and Hom-Ref carriers, respectively. CL increased by 40 (**1B*) and 48% (rs_4646437) per active allele of *CYP2B6* and *CYP3A4* as compared to the respective Hom-LOF. Further characterization of the joint contribution of *CYP2B6* and *CYP3A4* on EFV CL, using a mere addition of square root functions (Eq. 8), fitted the data appropriately, the loss of fit in comparison with previous models (Eqs. 5/6/7) being not significant (ΔOF = −9.0

Table 2 Summary of the key models used to examine the influence of demographic and genetic covariates on EFV clearance

Step 1	Demographic model	Model	θ_0	θ_1	θ_2	θ_3	ΔOF	P
	Body weight (BW)	$CL=\theta_0\cdot(1+\theta_1\cdot BW)$	11.5	1.2			−25.6	**
	Height	$CL=\theta_0\cdot(1+\theta_1\cdot Hgt)$	11.2	2.3			−4.2	*
	Age	$CL=\theta_0\cdot(1+\theta_1\cdot age)$	12	0.7			−5.7	*
	Sex (M = 0, F = 1)	$CL=\theta_0\cdot(1+\theta_1\cdot sex)$	12	0.2			−4.1	*
	Race	$CL=\theta_0(1-q)+\theta_1\cdot q$						
	Black ($q=1$) vs. others ($q=0$)		11.8	6.25			−13.2	**
	White ($q=1$) vs. others ($q=0$)		12	7.1			−7.7	**
	Hispanic ($q=1$) vs. others ($q=0$)		15.1	11.2			−0.9	NS
	Asian ($q=1$) vs. others ($q=0$)		11.3	9.8			−0.13	NS
	Other ARV							
	Ritonavir (RTV)	$\theta_0\cdot(1+\theta_1\cdot RTV)$	11.3	0.01			0.2	NS
	Zidovudine (AZT)	$\theta_0\cdot(1+\theta_1\cdot AZT)$	10.4	0.12			−1.1	NS
	Lamivudine	$\theta_0\cdot(1+\theta_1\cdot 3TC)$	10.6	0.09			−0.6	NS
	NRTI in general	$\theta_0\cdot(1+\theta_1\cdot NRTI)$	8.6	0.3			−1.8	NS
	PI in general	$\theta_0\cdot(1+\theta_1\cdot PI)$	11.3	0.01			0.6	NS
Step 2	**Genotype-variant analysis**	**Model**	CL_0	θ_1	θ_2	θ_3	ΔOF	P
CYP2B6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref, I_3: GOF	$CL=CL_0+\theta_1 I_1+\theta_2 I_2+\theta_3 I_3$	2.8	10.8	13.3	18.8	−171	**
Reduced 1	I_1: Het-LOF or Hom-Ref, I_3: GOF	$CL=CL_0+\theta_1 I_1+\theta_3 I_3$	2.8	12.2	—	18.9	−162	**,a
Reduced 2	I_1: Het-LOF, I_2: Hom-Ref or GOF	$CL=CL_0+\theta_1 I_1+\theta_2 I_2$	2.8	10.8	14.2		−161	**,a
Eq. 2	$n=0, 1, 2, 3$	$CL=CL_0+\theta_1\cdot n$	3.11	5.99			−152	**,a
Eq. 3		$CL=CL_0+\theta_1^n$	5.03	2.9			−76	**,a
Eq. 4		$CL=CL_0+\theta_1\cdot\sqrt{n}$	2.8	7.8			−168	NS[a]
CYP2A6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL=CL_0+\theta_1 I_1+\theta_2 I_2$	7.0	10.8	12.1		−7.9	*
Reduced 1	I_1: Het-LOF or Hom-Ref	$CL=CL_0+\theta_1 I_1$	7.0	11.7			−6.9	NS[a]
Eq. 2	$n=0, 1, 2$	$CL=CL_0+\theta_1\cdot n$	7.75	2.31			−6.8	NS[a]
Eq. 3		$CL=CL_0+\theta_1^n$	7.8	2.11			−5.3	NS[a]
Eq. 4		$CL=CL_0+\theta_1\cdot\sqrt{n}$	7.0	3.63			−7.8	NS[a]
CYP3A4 rs4646437								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL=CL_0+\theta_1 I_1+\theta_2 I_2$	3.7	10.7	12.3		−25.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL=CL_0+\theta_1 I_1$	3.7	11.9			−24	NS[a]
Eq. 2	$n=0, 1, 2$	$CL=CL_0+\theta_1\cdot n$	4.84	4.06			−20	*,a
Eq. 3		$CL=CL_0+\theta_1^n$	6.2	2.5			−12.9	*,a
Eq. 4		$CL=CL_0+\theta_1\cdot\sqrt{n}$	3.89	6.1			−24.8	NS[a]
*CYP3A4*1B*								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL=CL_0+\theta_1 I_1+\theta_2 I_2$	5.1	10.3	11.9		−10.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL=CL_0+\theta_1 I_1$	5.1	11.7			−9.5	NS[a]
Eq. 2	$n=0, 1, 2$	$CL=CL_0+\theta_1\cdot n$	6	3.6			−9.0	NS[a]
Eq. 3		$CL=CL_0+\theta_1^n$	6.4	2.3			−7.0	NS[a]
Eq. 4		$CL=CL_0+\theta_1\cdot\sqrt{n}$	5.2	4.8			−10.4	NS[a]
CYP3A5								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-LOF	$CL=CL_0+\theta_1 I_1+\theta_2 I_2$	4.4	10.3	11.8		−8.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-LOF	$CL=CL_0+\theta_1 I_1$	4.4	11.5			−7.4	NS[a]
Eq. 2	$n=0, 1, 2$	$CL=CL_0+\theta_1\cdot n$	6.15	2.96			−6.4	NS[a]
Eq. 3		$CL=CL_0+\theta_1^n$	7.0	2.22			−4.8	*,a
Eq. 4		$CL=CL_0+\theta_1\cdot\sqrt{n}$	4.7	5.2			−8.1	NS[a]

Table 2 Continued on next page

Table 2 (Continued)

Step 3	Gene–gene interaction analysis	*CYP* contribution	CL_0	θ_1	θ_2	θ_3	θ_4	ΔOF[a]
Eq. 5[b]	*CYP2B6/CYP2A6*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.8	2.59 7.42 9.74 14.6	3.15 11.6 11.9 19.6	10.9 14.3 18.8		−16
Eq. 6	*CYP2B6/CYP2A6*	*CYP2B6* *CYP2A6*·$\sqrt{q}$	1.75	8.15 0.95	2.42	9.21 3.44	19.3 2.49	−17
Eq. 7	*CYP2B6/CYP2A6*	*CYP2B6* *CYP2A6*·$\sqrt{q}$		2.02 1.21	7.7	9.45	13.6	−16
Eq. 8	*CYP2B6/CYP2A6*	*CYP2B6*·$\sqrt{p}$ *CYP2A6*·$\sqrt{q}$	1.5	7.7 1.2				−12
Eq. 5[b]	*CYP2B6/CYP3A4 _rs4646437*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.66	2.46 10.5 13.3 16.3	3.9 11 12.3 17.0	10.6 13.5 19.3		−25
Eq. 6	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6* *CYP3A4 _rs*·$\sqrt{r}$		1.6 1.4	10.7 0.1	11.2 1.6	15.3 2.6	−22
Eq. 7	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6* *CYP3A4 _rs*·$\sqrt{r}$		1.62 1.36	8.99	11.5	16.9	−22
Eq. 8	*CYP2B6/CYP3A4 _rs4646437*	*CYP2B6*·$\sqrt{p}$ *CYP3A4 _rs*·$\sqrt{r}$	1.62	7.3 1.34				−18
Eq. 5[b]	*CYP2B6/CYP3A4*1B*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.69	2.33 10.6 13.4 16.4	3.23 10.3 11.7 17.2	10.8 13.6 19.4		−14
Eq. 6	*CYP2B6/CYP3A4*1B*	*CYP2B6* *CYP3A4*1B*·$\sqrt{s}$		1.64 1.1	10.7 0.4	11.2 1.2	15.3 2.7	−13
Eq. 7	*CYP2B6/CYP3A4*1B*	*CYP2B6* *CYP3A4*1B*·$\sqrt{s}$		1.62 1.1	8.99	11.5	16.9	−12
Eq. 8	*CYP2B6/CYP3A4*1B*	*CYP2B6*·$\sqrt{p}$ *CYP3A4*1B*·$\sqrt{s}$	1.65	7.6 1.1				−9
Eq. 5[b]	*CYP2B6/CYP3A5*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.45	2.22 6.0 13.4 16.5	3.25 11.6 11.9 17.0	10.6 13.6 19.2		−16
Eq. 6	*CYP2B6/CYP3A5*	*CYP2B6* *CYP3A5*·$\sqrt{t}$		1.38 1.1	10.7 0.1	11 1.8	15 2.4	−14
Eq. 7	*CYP2B6/CYP3A5*	*CYP2B6* *CYP3A5*·$\sqrt{t}$		1.4 1.22	9.1	11.7	17.1	−13
Eq. 8	*CYP2B6/CYP3A5*	*CYP2B6*·$\sqrt{p}$ *CYP3A5*·$\sqrt{t}$	1.4	7.6 1.22				−10

ΔOF, difference in the objective function, compared to the final model structural model; ΔOF*, difference in the objective function compared to the model including CYP2B6 solely; ARV, antiretroviral medication; NRTI, non-nucleoside/nucleotide reverse transcriptase inhibitors; *n*, *p*, *q*, *r*, *s*, numbers of functional alleles (0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF); NS, not statistically significant; PI, protease inhibitors.

[a]Differences in objective function compared to the rich model (Eq. 1). [b]I_{xy} represents the number of functional alleles for the x/y cytochromes. For clarity, θ have been numbered from 1 to 4. *$P < 0.05$, **$P < 0.01$.

for **1B*, −18 for rs_4646437). No factor accounting for more than an additive interaction was observed (ΔOF = 0.0).

The joint assignment of *CYP2B6* and *CYP3A5* on CL also improved the fit as compared to *CYP2B6* alone (Eq. 5, ΔOF = −16). The successive nesting of models using functional scores successfully followed the paradigm previously described for other *CYPs*. The influence of *CYP3A5* on *CYP2B6* was appropriately described using Eqs. 6 or 7 (ΔOF >−13) or as a square root additive model (Eq. 8, ΔOF >−10). Again, an interaction between the two genes was not significant (ΔOF = 0.0).

A final joint model characterizing the cumulative influence of all the genetic variants on EFV CL was tested on the basis of the two-by-two combinations of genetic effects. The richest model that integrated the effects of all CYP alleles, using a generalization of Eqs. 6 and 7, improved the fit (ΔOF >−29 as compared to *CYP2B6* alone), but the impact of *CYP3A4*1B* and *CYP3A5* did not remain significant (ΔOF = 0.0 as compared to the rich model without those two alleles). The final model employed a single parameter estimate to quantify the influence of each of the alleles, *CYP2B6*, *CYP2A6*, and *CYP3A4 _rs4646437*, using additive square root functions, which fitted the data appropriately (Eq. 10, ΔOF = −24). All models specified with proportional rather than additive effects gave very similar results (ΔOF <−0.5). In addition to genetic influences on CL, the influence of body weight remained the only significant demographic covariate (ΔOF = 19.2), while any ethnic influence vanished completely.

The final model estimated an average CL of 1.3 l/h in individuals carrying Hom-LOF *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437, which increased by 7.3, 0.7, and 1.03 l/h for the first active allele of those genes, respectively, and by another 0.41 times those factors for the second allele (where $0.41 = \sqrt{2} - 1$), with an additional 70% increase in CL associated with doubling of body weight. A summary of the model building procedure is presented in Table 2 and the final population estimates in Table 3. A plot of the model-predicted concentration profile stratified by *CYP2B6* is shown in Figure 2. Figure 3 represents the individual *post hoc* CL estimates with population average predictions for the different allelic combinations encountered.

Based on our final model, simulations show that, with the standard regimen of 600 mg of EFV daily, average trough concentrations are 1.19 (90% prediction interval: 0.6–2.35), 1.6 (0.8–2.5), and 8.1 mg/l (4.5–14.5) in *CYP2B6* Hom-Ref, Het-LOF, and Hom-LOF carriers, respectively, and 0.5 mg/l (0.2–1.0) in *CYP2B6* Het-GOF carriers. Taking into account the interindividual variability leads to the suggestion that most *CYP2B6* Hom-LOF individuals will exhibit concentrations exceeding the 1–4 mg/l range that is generally considered acceptable, and most individuals carrying a Het-GOF will have concentrations <1 mg/l. Predicted concentrations of 2.7 mg/ml (1.5–4.9) would be expected at a dosage regimen of 200 mg/day in *CYP2B6* Hom-LOF carriers. However, individuals having Hom-LOF of *CYP2A6*/*CYP3A4_* rs3434367 and *CYP2B6* will be exposed to considerably higher drug levels, yielding average predicted concentrations of 6.2 mg/ml (3.5–11.0) with a regimen of 200 mg/day.

Table 3 Final population pharmacokinetic parameter estimates of EFV

	Population mean		Interindividual variability	
Parameter	Estimate	SE (%)[a]	Estimate (%)[b]	SE (%)[c]
CL/F (l/h)[d]	1.3	18	27.8	43.8
θ_{2B6}[e]	7.3	7		
θ_{2A6}[e]	0.7	36		
$\theta_{3A4\_rs4646437}$[e]	1.03	27		
θ_{BW}[f]	0.7	42		
V_d/F (l)	332	16		
k_a (h^{-1})	0.6	38		
σ (CV%)[g]	30.8	44.2		

CL/F, mean apparent clearance; F, bioavailability; k_a, mean absorption rate constant; V_d/F, mean apparent volume of distribution.

[a]SEs of the estimates (SEE), defined as SE/estimate and expressed as percentages. [b]Estimate of variability is expressed as coefficient of variation (CV) (%). [c]SEs of the CV, taken as $\sqrt{(SE/estimate)}$ and expressed as percentage. [d]CL value in patients with Hom-LOF for *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437. [e]Contribution of *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437 to efavirenz (EFV) CL multiplied by $\sqrt{n}$ where $n = 0, 1, 2, 3$ for *CYP2A6*, $n = 0, 1, 2$ for *CYP2A6* and *CYP3A4_*rs4646437 (see text). [f]Relative influence of body weight on EFV clearance (see text). [g]Residual intrapatient variability, expressed as a CV (%).

DISCUSSION

This study enabled us to characterize and quantify for the first time the conjugated effects of major and minor metabolic pathways and their genetic variants on EFV pharmacokinetics in a population of HIV-1-infected patients. The average CL and variability of EFV plasma concentrations are in the range of values reported previously.[1–3] Among nongenetic covariates, we observed an impact of body weight on CL, as reported by others.[16–20] In our study, no differences could be observed between data from male and female subjects, but conflicting results exist in the literature.[20–23] The well-known influence of black ethnicity, which has been associated with *CYP2B6* and *CYP3A4* heterogeneity,[24,25] could

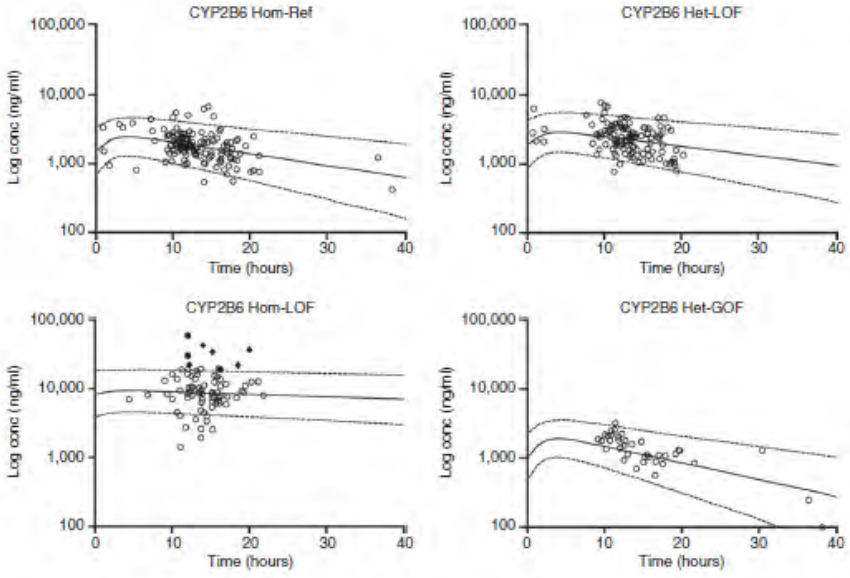

Figure 2 Efavirenz (EFV) plasma concentrations ($n = 393$) in 169 type I human immunodeficiency virus (HIV-1) individuals (open circles) in relation to *CYP2B6* polymorphism. Population predictions of the corresponding genotype are represented by black lines, and the 90% prediction interval is shown by gray dotted lines. Left lower panel: filled circles represent concentrations in individuals who are Hom-LOF for CYP2B6, CYP2A6, and CYP3A4_rs46464337, and filled diamonds represent concentrations in individuals who are Hom-LOF for CYP2B6 and Het-LOF for CYP3A4_rs4646437. Het, heterozygous; Hom, homozygous; LOF, loss of function.

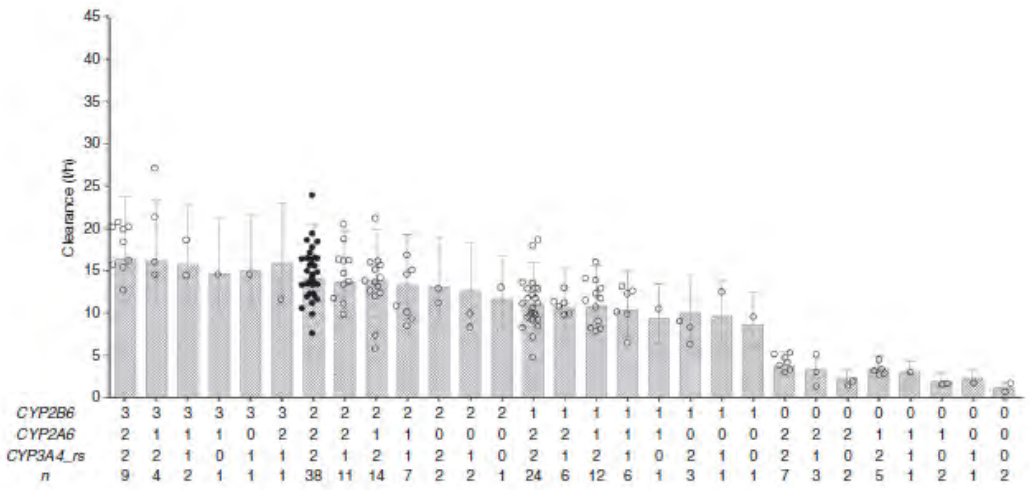

Figure 3 Individual predicted Bayesian clearances (open circles, the black circles correspond to individuals with reference alleles for all three cytochromes) and average predicted clearances (bars) with 90% population prediction interval for each *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437 allelic combination (according to score A categorization: 0 Hom-LOF/DOF, 1 Het-LOF/DOF, 2 Hom-Ref, and 3 Het-GOF; see text); *n* = number of individuals carrying the allelic combination (*n* = 0 for the eight combinations that are not represented). DOF, diminished function; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

be mostly explained by the joint influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* variations, whereas its influence remained discernible when these *CYP* alleles were considered separately. Given the limited number of Hispanic ($n = 6$) and Asian ($n = 5$) individuals in our study, no clear effect on EFV levels could be attributed to these ethnic groups, although an influence has been reported previously.[1,3,10,20] Among genetic covariates, *CYP2B6* allelic variation accounted for most of the interindividual differences in EFV CL. The *CYP2A6* and *CYP3A4/A5* accessory pathways appeared to influence EFV elimination independent of *CYP2B6*. Among those, the overall impact of *CYP3A4*_ rs4646437 was the largest; it accounted for 6% of CL variability. The unexpected lower CL in *CYP3A5* Hom-Ref carriers as compared to Hom-LOF carriers can be explained by the linked Hom/Het-LOF of *CYP2B6* and *CYP3A4*_ rs4343437 observed in most individuals. This hypothesis is further confirmed by the lack of effect of *CYP3A5* when the influence of all cytochromes is assessed in a joint analysis. When information on *CYP2B6* was included along with *CYP2A6* and *CYP3A4* in joint analyses, an additive effect of accessory pathways was still present, both on fully functional *CYP2B6* and in the presence of reduced-function or gain-of-function alleles. However, the compensation ensured by these *CYP* alleles was small (1–2 l/h) and therefore more discernible on *CYP2B6* Hom-LOF. An evaluation of *CYP* genetic variations according to an activity score, as recently proposed by Gaedigk *et al.*[26] for *CYP2D6*, enabled us to quantify genetic influences of all alleles based on the same paradigm. A model of remarkable parsimony, which included only one parameter per allele and required no interaction term, could capture the nonlinear relationship between CL and the different genotypic groups. The use of such square root relationships in gene–dose effects was not described for other *CYP* isoenzymes, in particular for the noninducible CYP2D6,[24] which tend more toward linear effects.[27] This phenomenon suggests adaptive mechanisms that might be explained by the upregulation of extensive metabolizer alleles in response to an increase in concentration, possibly through the activation of nuclear receptors.[28,29] It is noteworthy that a similar pattern has already been reported for *CYP2B6*, not only in relation to EFV[30] but also in relation to S-methadone.[31] The use of different functional score classifications to implement partial activity levels failed to improve the model when compared with the traditional classification. The mechanisms by which allelic variants express a loss/diminished function could therefore not be translated in a "semiquantitative gene–dose system," as described by Steimer *et al.*[32] for amitriptyline and nortriptyline.

The cumulative influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437 allelic variants on CL implies a critical 90% decrease of EFV elimination in triple-Hom-LOF individuals, in whom CL appeared reduced to 1.3 l/h as compared to the estimated 12.9 l/h in triple-Hom-Ref individuals. Of note, the only individual who exhibited extremely high EFV concentrations[33] was found to be triple-Hom-LOF; this emphasizes the importance of accessory pathways in EFV elimination. The interindividual variability in CL dropped from 65 to 27% in the final model, with *CYP2B6* genetic variants accounting for 31% and another 7% being explained by *CYP2A6*, *CYP3A4*_ rs4646437, and body weight variations. The remaining variability might be attributed to adherence issues[34] or to variation in uridine-glucuronyltransferase metabolic pathways.

In conclusion, functional alleles of *CYP2B6* accounted for the major part of EFV interindividual variability. Genetic variations in EFV accessory metabolic pathways demonstrated their importance in EFV pharmacokinetics in addition to *CYP2B6*, in particular in individuals with limited *CYP2B6* function. The dosage is required to be reduced to 200 mg/day of EFV in individuals with impaired *CYP2B6* function so as to ensure drug levels within the therapeutic range. The expression of pharmacogenetic influences on EFV elimination could be characterized using a single common paradigm across all *CYPs*. This paradigm involves the addition of the contribution of each enzymatic pathway proportional to the square root of the number of

Table 4 Demographic and genetic characteristic of the population study

Characteristics	Value	% Of study population
Sex (no.)		
Men	124	73
Women	45	27
Age (years)		
Median (range)	47 (30–73)	—
Body weight (kg)		
Median (range)	77.5 (44–101)	—
Height (cm)		
Median (range)	179 (153–193)	—
Ethnicity (no.)		
White	142	83
Black	16	10
Hispanic	6	4
Asian	5	3
PIs (no.)		
Ritonavir	20	13
Saquinavir	4	3
Amprenavir	0	0
Lopinavir	15	9
Atazanavir	18	11
NRTIs (no.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Abacavir	0	0
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (no.)		
Enfuvirtide	4	2
CYP P450 inducers (no.)	2	1
CYP P450 inhibitors (no.)	4	2
CYP2B6 *genetic polymorphism (no.)*		
Hom-Ref	75	44
Het-LOF	53	33
Hom-LOF	23	14
Het-GOF	16	9
Het-LOF/Het-GOF[a]	2	1
CYP2A6 *genetic polymorphism (no.)*		
Hom-Ref	99	61
Het-LOF	55	30
Hom-LOF	13	8
Het-GOF	2	1
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	138	82
Het-LOF *1B	24	14
Hom-LOF *1B	7	4
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	118	70
Het-LOF_rs4646437	41	24
Hom-LOF_rs4646437	10	6
CY3A5 *genetic polymorphism (no.)*		
Hom-Ref	5	3
Het-LOF	30	18
Hom-LOF	134	79

GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; Ref, reference allele.

[a]Two individuals are LOF/GOF for *CYP2B6* and were considered to be Het-LOF.

functional alleles. Such a model appropriately predicts average CL for various allele combinations, and its mechanistic explanation warrants further investigation.

METHODS

Study population. A total of 169 HIV-1-infected individuals from the Swiss HIV Cohort Study were characterized with respect to *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. EFV drug levels were measured during routine therapeutic drug monitoring according to local treatment guidelines. All participants gave their informed consent for genetic testing. A median of 1 concentration sample per individual (range 1–23) was collected between 0.6 and 38 h after the last drug intake under steady-state conditions.

Method of analysis. Blood samples (5 ml) were collected into lithium heparin or EDTA-K Monovette syringes (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation, inactivated for virus at 60 °C for 60 min, and stored at −20 °C until analysis. Plasma EFV levels were determined by liquid chromatography coupled with tandem mass spectrometry in accordance with a validated method. The calibration curves were found to be linear up to 10 µg/ml, with a lower limit of quantification of 0.1 µg/ml.

Nomenclature and functional score. Alleles are designated in concordance with the *CYP* Allele Nomenclature Committee (http://www.cypalleles.ki.se). Proposed functional consequences for EFV pharmacokinetics have been reported for *CYP2B6*,[15] *CYP2A6*, and *CYP3A4/A5*.[35] The study participants were categorized into genotypic groups on the basis of the number of functional alleles (Table 1). The simplest scoring scheme (score A) assigned values of 2, 0, and 1 to the fully functional reference (Hom-Ref), homozygous (Hom-LOF), and heterozygous (Het-LOF) diminished/loss of function, respectively, and a value of 3 to *CYP2B6* gain-of-function alleles (Het-GOF). Two individuals having a single-gene duplication of *CYP2A6* were assimilated to the Hom-Ref group because they constituted only a small number. The classification was refined to distinguish between individuals with *CYP2B6* and *CYP2A6* loss/diminished function alleles, to reflect the predicted level of activity from *in vitro* studies (Table 1, scores B/C).

Pharmacokinetic structural model. EFV pharmacokinetics were characterized using a one-compartment model, as assessed previously.[2] Since EFV is administered only orally, CL and *V* represent apparent values.

Covariate model. The analyses of the covariate effects on CL were divided into three main sections for assessing: (i) the influence of demographic variables and concomitant medications, (ii) the impact of *CYP2B6*, *CYP2A6*, *CYP3A4*, and *CYP3A5* alleles based on univariate analyses, and (iii) the joint effect of *CYP2B6* with *CYP2A6*, *CYP3A4*, and *CYP3A5* alleles in multivariate analyses.

Demographic analyses. The typical value of CL was modeled to depend linearly on a covariate X (body weight, centered on the mean; categorical covariates coded as 0/1) as shown in the equation: $CL = \theta_a \cdot (1 + \theta_b \cdot X)$, where θ_a is the average estimate and θ_b is the relative deviation (positive or negative) from average attributed to the covariate X. The available demographic covariates were sex, ethnicity, age, body weight, and height. Only a few co-medications were recorded, and these were principally other antiretroviral drugs and known *CYP* inducers or inhibitors (Table 4).

Univariate genotype. In these analyses, each genotype was entered solo into the model. Several models relating CL with functional scores were tested using different methods (Figure 1) and compared with the richest possible model, which assigned a separate fixed effect to each score level as follows:

$$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3 \quad (1)$$

$$CL = CL_0 \cdot (1 + \theta_1 I_1) \cdot (1 + \theta_2 I_2) \cdot (1 + \theta_3 I_3) \quad (1a)$$

where CL_0 is the typical value of CL in Hom-LOF individuals (Hom-Ref for *CYP3A5*), I_i is an indicator variable that takes the value of 1 if an individual carries the ith genotypic score (i.e., I_1: Het-LOF, I_2: Hom-Ref, and I_3: Het-GOF) and 0 otherwise, and θ_i is the absolute or fractional (Eqs. 1/1a) change in CL relative to the Hom-LOF group. The impact of functional alleles on EFV CL was further explored to distinguish the difference between the genotypic groups, using two reduced models in which the same genotyping group was assigned to Hom-Ref and Het-LOF or to Hom-Ref and Het-GOF carriers (reduced 1 and 2, Figure 1). Competing models attempted to account for gene effect as a function of the number of functional alleles (Table 1, score A), using linear and power relationships with either additive or proportional (data not shown) impact, using the following models:

$$CL = CL_0 + \theta_1 \cdot n \quad (2)$$

$$CL = CL_0 + \theta_1^n \quad (3)$$

$$CL = CL_0 + \theta_1 \sqrt{n} \quad (4)$$

where $n = 1$, 2, or 3 represents the functional score, and θ_1 the average contribution per active allele above that of Hom-LOF CL (CL_0). The alternative activity scores B/C for *CYP2B6* and *CYP2A6* were explored using parameter models in Eqs. 2, 3, or 4 and compared with an extension of the rich model (Eq. 1).

Gene–gene interaction analyses. The joint influence of functional alleles on EFV CL was first tested using pairwise conjunction of *CYP2B6* with each of the other *CYP* alleles, so as to finally build up a model including all genetic variants that have an influence. The investigation of the joint influence of *CYP2B6* and *CYP2A6* alleles is shown as an example. The richest model that served as reference for the evaluation of reduced competing models was:

$$\begin{aligned} CL = {} & CL_0 + \theta_{01} I_{01} + \theta_{02} I_{02} + \theta_{10} I_{10} + \theta_{11} I_{11} + \theta_{12} I_{12} \\ & + \theta_{20} I_{20} + \theta_{21} I_{21} + \theta_{22} I_{22} + \theta_{30} I_{30} + \theta_{31} I_{31} + \theta_{32} I_{32} \end{aligned} \quad (5)$$

where CL_0 is the Hom-LOF CL for both genes and I_{ij} is an indicator variable that takes the value of 1 for the *CYP2B6* ith/*CYP2A6* jth genotype carrier and is 0 otherwise, and each θ_{ij} estimates the absolute change in CL among the different genotypic groups. The same model was parameterized for relative changes (data not shown). The following competing models were evaluated:

$$\begin{aligned} CL = {} & CL_0 + \theta_{0\_0} \cdot \sqrt{q} + \left(\theta_1 I_1 + \theta_{1\_1} \cdot \sqrt{q}\right) \\ & + \left(\theta_2 I_2 + \theta_{2\_2} \cdot \sqrt{q}\right) + \left(\theta_3 I_3 + \theta_{3\_3} \cdot \sqrt{q}\right) \end{aligned} \quad (6)$$

$$CL = CL_0 + (\theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3) + \theta_4 \cdot \sqrt{q} \quad (7)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} \quad (8)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot (p \cdot q) \quad (9)$$

where p indicates the functional score for *CYP2B6* and q the score for *CYP2A6*. In Eq. 6, the contribution of *CYP2A6* (θ_{0_0},...,θ_{3_3}) is investigated on *CYP2B6* stratified by genotypic groups; in Eq. 7 the influence of *CYP2A6* is characterized using a single fixed-effect parameter θ_4 across all *CYP2B6* genotypes; and in Eq. 8 square root functions are integrated for both genes. Finally, an interaction term was allowed so as to further check for some nonadditive interaction between *CYP* variants (Eq. 9). All these models used either additive or proportional (data not shown) effects.

All significant allelic groups were integrated into a final model, wherein the contribution of each genotypic group was estimated using a generalization of Eqs. 6–8 to be finally formulated using the following additive or proportional (data not shown) relationships:

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot \sqrt{r} + \theta_4 \cdot \sqrt{s} + \theta_5 \cdot \sqrt{t} \quad (10)$$

where CL_0 is the Hom-LOF CL for all genes and θ_i values estimate the absolute or fractional change in CL as a function of score A for different combinations of *CYP2B6* (p), *CYP2A6* (q), *CYP3A4* _rs4646437 (r), *CYP3A4*1B* (s), and *CYP3A5* (t) alleles.

Variance model. The individual CL values were modeled assuming a log-normal distribution (mean zero and variance Ω). A proportional error model (mean zero and variance σ^2) was used the description of intraindividual variability.

Parameter estimation and selection. NONMEM (version VI; NM-TRAN, version II, GloboMax, Hanover, MD) was used with FOCE INTERACTION to fit the models.[36] As goodness-of-fit statistics, NONMEM uses the objective function, which is approximately equal to minus twice the logarithm of the maximum likelihood. The likelihood ratio test, based on the reduction in objective function (ΔOF), was used to carry out comparisons between any two models. A ΔOF (−2 log likelihood, approximate χ^2 distribution) of 3.84, 5.99, and 7.81 points for 1, 2, or 3 additional parameters, respectively, was used for determining statistical significance ($P < 0.05$) of the difference between two models. The reliability of the results was checked on diagnostic goodness-of-fit plots, along with the measure of the SEs. The identification of potential outlier values resulting from compliance issues or inadequacy in self-reporting information was explored using a sensitivity analysis. One individual had an EFV concentration of 59,400 ng/ml, and this value was at first excluded to prevent single outlier effect but was integrated at the end. Except for this, all data were considered reliable. Simulations based on the final pharmacokinetic estimates were performed with NONMEM using 1,000 individuals to calculate the 90% prediction intervals. The concentrations encompassing the range from 5th to 95th percentile at each time point were retrieved in order to construct the intervals. Further simulations were performed for a series of genotype combinations in 1,000 individuals undergoing various dosage regimens (200, 400, 600, and 800 mg), so as to suggest the dosages that would ensure trough levels falling within the 1–4 mg/ml therapeutic interval in 90% of the individuals. The figures were generated using GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA, http://www.graphpad.com).

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M.A.-A. contributed to the pharmacokinetic modeling and J.D.I. to the genetic testing.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Protocole d'isolement des PBMCs (peripheral blood mononuclear cells) à partir de filtres du centre de transfusion sanguine

Produits chimiques :

-	Tampon PBS	env. 260 ml	RAFA n° C01768
-	Solution RPMI-1640 Medium	60 ml	RAFA n° C03425
-	Solution Ficoll (Biocoll solution séparation)	60 ml	RAFA n° C04066
-	FBS (fœtal bovine serum)	4 ml	RAFA n° C07066

Équipements :

		pour 2 filtres	
-	Ciseaux	1	
-	Seringue 60.0 ml	1	RAFA n° 736084
-	Flacon FALCON culture tissus stérile T75	2	RAFA n° 317007
-	Tube Falcon 50 ml	6	
-	Pipettes PPE 10.0 ml	10	RAFA n° 316672
-	Pipettes PPE 25.0 ml	10	RAFA n° 311303
-	Pipettes Pasteur en plastique	X (entre 20-30)	
-	Tubes Eppendorf 1.5 ml	X (entre 20-30)	
-	Portoir pour tubes Eppendorf	1	
-	Portoir pour tubes Falcon	1	
-	Becher de 150 ml	1	
-	Centrifugeuse Beckmann® Model J-6B		
-	Centrifugeuse Hettich® Benchtop Universal 16R		
-	Pipetboy		

Normes de sécurité :

Les manipulations avec du matériel biologique potentiellement infectieux (sang, plasma) se font toujours avec des gants de protection.

Solutions :

- Solution RPMI/FBS : solution RPMI additionné de 2% de FBS

Mode opératoire :

Le jour avant :

- Téléphoner au centre de transfusion sanguine (n° interne : 46580) pour commander les filtres pour la PCL (donner le nom de Laurent Decosterd). En général 2 filtres sont commandés.
  (*Pour le lendemain matin* : poches de la veille filtrées le matin même ; *Pour le lendemain après-midi* : poches du jour filtrées directement).

Le jour de la préparation :

- Chercher les filtres à la transfusion (CTS, ch. De la Corniche 2, 1066 Epalinges ; sortie M2 Croisettes), en s'étant assuré qu'ils sont prêts (si nécessaire retéléphoner le matin même).

Récolte des PBMC :

- Couper les extrémités des tubes du filtre avec des ciseaux.
- Mettre 100 ml de PBS dans le becher, afin de pouvoir aspirer la solution avec la seringue.
- Placer la seringue de 60.0 ml remplie d'environ 30 ml de PBS à l'extrémité du tube inférieur (tube B, *figure 1*) et placer l'extrémité du tube supérieur (tube A, *figure 1*) dans la boite T75 contenant préalablement 30 ml de solution RPMI additionné de 2% FBS.
- Rincer le filtre avec la solution PBS, puis faire passer un peu d'air au travers du filtre pour faire sortir le reste de sang.
- Dans des tubes Falcon de 50.0 ml (2 tubes/filtre), mettre 15 ml de solution Ficoll à température ambiante, et y ajouter 30 ml de mélange sang/PBS en laissant couler lentement le mélange contre la paroi du tube placé presque horizontalement (Attention à **ne pas mélanger les deux phases** !).
- Centrifuger les 4 tubes Falcon pendant 30 minutes à 300 g (1100 rpm) sur la centrifugeuse Beckmann® sans mettre de frein.
- Collecter les anneaux de cellules à l'aide d'une pipette Pasteur (*figure 2*) et mettre ces cellules dans un tube Falcon de 50.0 ml (1 tube/filtre). Le volume est ajusté à 50 ml avec la solution PBS pour le premier lavage des cellules.
- Centrifuger le tube pendant 10 minutes à 540 g (2000 rpm) en utilisant le frein (maximum), éliminer le surnageant et répéter le lavage deux fois avec 20 ml de solution PBS.
- Après la dernière centrifugation, éliminer complètement le surnageant et resuspendre les cellules dans environ 30 ml (ou moins selon le nombre de cellules par ml désiré) de solution PBS de façon la plus homogène possible.
- Prélever 1 aliquot de 100µl dans un Eppendorf (1.5 ml) et ajouter 300µl de Diluant Cellpack afin d'atteindre un volume final de 400µl. Les 2 aliquots sont utilisés pour le comptage cellulaire (« numérotation ») qui doit être effectué dans les minutes qui suivent par le laboratoire d'hématologie (Bon LCH n° 23).
- Distribuer ensuite la suspension de cellules en X aliquots de Y ml chacun dans des tubes Eppendorf (1.5 ml ou 2.0 ml) selon le comptage cellulaire effectué et le nombre de cellules voulu dans chaque culot au final (attention aux unités et facteur de dilution avec le Cellpack !). Centrifuger les tubes pendant 10 minutes à 20'000 g (14'000 rpm) à 4°C à l'aide de la centrifugeuse Hettich®.
- Jeter le surnageant dans une poubelle pour les liquides biologiques et conserver les culots de cellules dans les tubes Eppendorf à - 20°C, en veillant à bien indiquer sur l'Eppendorf combien de cellules contient chaque culot.

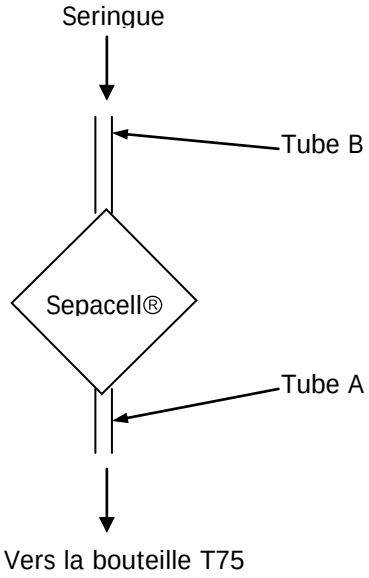

Figure 1 : Position du filtre

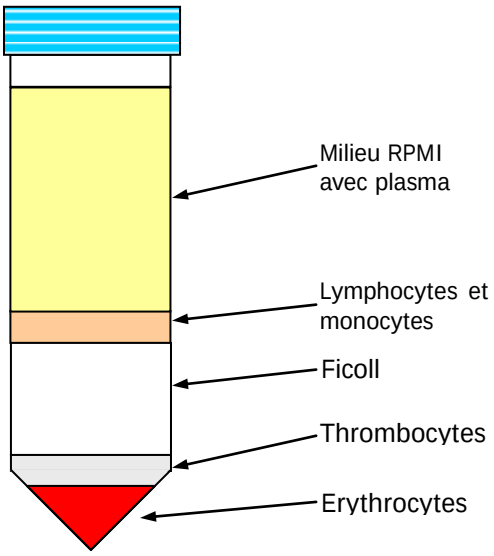

Figure 2 : Tube Falcon après centrifugation

Protocole d'isolement des PBMCs (peripheral blood monocyte cells) à partir de tubes vacutainer CPT®

Produits chimiques :

- Tampon PBS, 500mL — Sigma-Aldrich (RAFA : C01768)
- FBS (foetal bovine serum) Heat Inactivated, 500mL — Invitrogen (RAFA : C07066)
- Cellpack Diluant, pour comptage cellulaire — A demander au labo Hématologie (A. Vienny Grob)

Équipements :

- Vacutainer CPT citrate de Na 8mL — Becton Dickinson (RAFA : 241517)
- Bac de glace
- Tube Falcon 50 ml
- Tubes Falcon 15 ml
- Pipettes 10 ml pour pipetboy
- Pipettes 2 ml pour pipetboy
- Pipetboy
- Pipettes Pasteur plastiques
- Tubes Eppendorf 1.5 ml
- Tubes Eppendorf 0.5 ml
- Portoir pour tubes Eppendorf
- Centrifugeuse Beckmann® Model J-6B
- Centrifugeuse Hettich® Benchtop Universal 16R

Normes de sécurité :

Les manipulations avec du matériel biologique potentiellement infectieux (sang, plasma) se font toujours avec des gants de protection.

Solutions nécessaires :

- Solution tampon PBS 15 ml (dans tube Falcon conservé au frigo, puis sur de la glace)
- Solution tampon PBS/FSB 50 ml (dans tube Falcon conservé au frigo durant 3 semaines maximum, puis sur de la glace) : tampon PBS additionné de 5% de FBS

Mode opératoire :

Prélèvement :

- Noter précisément sur le bon l'heure du prélèvement sanguin.
- Remplir **complètement** le tube CPT avec le sang. Une fois que le tube est plein, attendre environ 3 secondes avant d'arrêter le prélèvement, afin d'assurer un remplissage correct du tube CPT® (c'est-à-dire 8.0 ml).
- Tout de suite après le prélèvement, retourner gentiment le tube une dizaine de fois afin de repartir de façon homogène l'anticoagulant présent dans le tube.
- La centrifugation doit être effectuée dans les minutes qui suivent l'homogénéisation.

Séparation des PBMC :

- Lors de la réception du tube, il est nécessaire de contrôler que les données inscrites sur le tube CPT et sur le formulaire sont correctes. Vérifier notamment l'absence de tout caillot de sang.
- Centrifuger le tube pendant **20 minutes** à 1650 g (sans frein et à **température ambiante**) sur la centrifugeuse Beckmann® (i.e. **2800 rpm**).
  [*Compter en plus 10 min jusqu'à l'arrêt complet de la centrifugeuse*]
- Sortir le tube de la centrifugeuse et l'agiter délicatement afin de redisperser l'anneau de PBMCs dans le plasma.
- Mettre la centrifugeuse Beckmann® à 4°C en déplaçant les aiguilles (verte et rouge).
- Verser le plasma contenant les PBMCs dans un tube Falcon de 15 ml qui a été préalablement placé dans de la glace (afin d'arrêter tout processus enzymatique)!
- Centrifuger le tube Falcon **10 minutes** à 650 g (i.e. **2000 rpm**) à **4°C** (toujours sans frein!) sur la centrifugeuse Beckmann®.
  [*Compter en plus 5 min jusqu'à l'arrêt complet de la centrifugeuse*]
- Éliminer le surnageant et rajouter 10 ml de PBS + 5% FBS (préalablement maintenu dans de la glace à 4°C), mélanger à l'aide de la pipette de 10 ml et du pipetboy pour resuspendre les cellules, puis centrifuger le tube Falcon **10 minutes** à 650 g (**2000 rpm**) à **4°C**.
- Répéter encore ce lavage à deux reprises.
- Après la dernière centrifugation, éliminer complètement le surnageant à l'aide d'une pipette pasteur et re-suspendre les cellules dans 1.0 ml de PBS 4°C de façon la plus homogène possible.
- Transférer cette suspension dans un tube Eppendorf **A** (1.5 ml) et prélever 1 aliquot de 50µl dans un autre Eppendorf **B** (0.5 ml) pour le comptage cellulaire qui doit être effectué dans les minutes qui suivent par le laboratoire d'hématologie.
- Ajouter 150µl de Diluant Cellpack à l'aliquot de 50µl contenu dans le tube **B** afin d'atteindre un volume final de 200µl, qui sera utilisé pour le comptage cellulaire.
- Le reste du volume du tube **A** est centrifugé (**10 minutes** à 650 g, **4°C** à l'aide de la centrifugeuse Hettich®, i.e. à **2900 rpm**) afin d'éliminer le PBS (conservé dans un autre Eppendorf pour analyse de contrôle). Le culot cellulaire contenu dans le tube est congelé (-20°C).

<u>Analyse par MS</u> :

Le médicament intracellulaire est extrait du culot de PBMCs congelés et dosé par LC-MS/MS.

<u>Comptage cellulaire (Coulter instrument)</u> :

- Labo Hématologie (JCH) au BH18
- Bon LCH n°23 : demander « FFS » : formule sanguine simple

<u>Exemple de calcul pour déterminer le nombre de cellules dans notre culot</u> :

- Nbre GB (comptage) = 0.347×10^9 cell/L = 0.347×10^6 cell/mL
- Dans 200 µL (volume de dilution pour le comptage), on a donc : 0.0694×10^6 cellules
- Dans 50 µL (volume de la suspension de cellules), on a donc : 0.0694×10^6 cellules
- Donc dans 1 mL (volume de resuspension de cellules), on a : 1.388×10^6 cellules
- On doit encore soustraire la quantité de cellules utilisées pour le comptage (50 µL). Donc au final on obtient :

$$1.388 \times 10^6 \text{ cellules} - 0.0694 \times 10^6 \text{ cellules} = \underline{1.319 \times 10^6 \text{ cellules}}$$

Protocole de dosage de raltégravir, maraviroc, darunavir, étravirine et ritonavir dans les PBMCs

Produits chimiques :

- Méthanol (MeOH)
- Acétonitrile (ACN)
- Eau Ultrapure MilliQ (H_2O)
- Acide formique (FA)
- Ammonium acetate

Equipements :

- Tubes Eppendorf + portoirs
- Hotte d'aspiration (chapelle)
- Pipettes *Eppendorf* P20, P200 , P1000 et P5000 et les embouts correspondants
- Vortex
- Agitateur
- Centrifugeuse Hettich universal 16R pour tubes Eppendorf
- Vials en verre pour HPLC (Hewlett Packard)
- Caps pour vials HPLC (Hewlett Packard)
- HPLC MS/MS
- Colonne Waters Atlantis^{TM}-dC18 3µm 2.1x50mm

Normes de sécurité :

Les manipulations avec du matériel biologique potentiellement infectieux (plasma) se font toujours avec des gants de protection.
Les manipulations avec les solvants se font sous une hotte d'aspiration avec des gants et si nécessaire (absence de protection vitrée) en portant des lunettes de protection.

Solutions mères :

Darunavir : 1 mg/mL dans MeOH
Etravirine : 1mg/mL dans EtOH + FA (1 goutte)
Raltegravir : 2 mg/mL dans MeOH/H_2O 1:1
Maraviroc : 0.2 mg/mL dans MeOH
Ritonavir : 1 mg/mL dans MeOH

Standard interne :

Darunavir-d_9 : 1 mg/mL dans MeOH

Préparation de la solution d'extraction (avec I.S.) :

15 mL solution mère DRV-d_9 dans 200mL MeOH/H_2O 1:1
Préparation des calibrateurs et contrôles de qualité

Préparation des calibrateurs et contrôles de qualité :

Les droites de calibration se font sur 7 points et sur un intervalle de 0.1 ng/mL à 80 ng/mL.

Solution X (10'000 ng/mL) :
50 µL RAL + 100 µL ETV + 100 µL DRV + 500 µL MVC + 100 µL RTV + 9150 µL solution d'extraction

Solution Y (1'000 ng/mL) :
200 µL solution X + 1800 µL solution d'extraction

Solution	Volume [µL]	Solution utilisée	Sol. extraction [µL]	Volume final [µL]	Concentration finale [ng/mL]
A	160	Y	1'840	2'000	80
B	100	Y	1'900	2'000	50
C	250	A	1'750	2'000	10
D	200	B	1'800	2'000	5
E	200	C	1'800	2'000	1
F	200	D	1'800	2'000	0.5
G	200	E	1'800	2'000	0.1
K	200	F	1'800	2'000	0.05
L	100	F	1'900	2'000	0.025
H	150	Y	1'850	2'000	75
I	50	Y	1'950	2'000	25
J	200	I	1'800	2'000	2.5

Conservation :

Les solutions pour Cs et QCs sont conservées à -20°C et mises à température ambiante (RT) avant utilisation.

Extraction :

- Ajouter 200 µL de solution pour Cs ou QCs (A → J) dans les tubes Eppendorf contenant les culots de PBMCs blancs dégelés.
- De manière similaire, ajouter 200 µL de solution d'extraction aux culots de cellules des patients.
- Vortexer la suspension pendant 30 sec. et soniquer pendant 10 minutes.
- Vortexer à nouveau 10 sec. pour s'assurer que les cellules sont décollées.
- Extraire pendant 30 minutes les échantillons en utilisant un agitateur.
- Centrifuger les échantillons pendant 10 minutes à 14'000 rpm (20'000 g) à 20°C.
- Introduire les 200 µL de surnageant dans un vial d'injection et fermer les vials.
- Injecter 20 µL.

Paramètres de l'analyse HPLC-MS/MS :

Méthode :

`RAL_DRV_MVC_ETV_RTV_intracell_090713.meth`

Colonne :

Waters Atlantis™-dC18 3µm 2.1x50mm
Phase mobile

Phase mobile :

-solvant A : 20mM Ammonium acetate dans H_2O + 0.1% acide formique
-solvant B : ACN + 0.1% acide formique

Détection :

Drug	Transition	CE	Q1 PW	Q3 PW	TL
Raltegravir	445.2 → 109.1	40	0.70	0.70	79
Maraviroc	514.4 → 280.1	37	0.70	0.70	86
Darunavir	548.2 → 392.2	20	0.70	0.70	64
Etravirine	435.0 → 304.0	40	1.50	1.50	112
Ritonavir	721.4 → 296.0	26	0.70	0.70	96
Darunavir-d_9	557.3 → 401.2	25	0.70	0.70	67

Source CID Collision Energie : 10

Gradient :

Time	Solvant A %	Solvant B %	Débit µL/min
0.00	98.0	2.0	300.0
1.00	98.0	2.0	300.0
3.20	70.0	30.0	300.0
10.00	0.0	100.0	300.0
10.10	0.0	100.0	500.0
15.00	0.0	100.0	500.0
15.10	98.0	2.0	400.0
17.50	98.0	2.0	400.0
18.00	98.0	2.0	300.0
20.00	98.0	2.0	300.0

Temps de retention :

Maraviroc 5.15 min
Raltegravir 6.30 min
Darunavir 7.00 min
Darunavir-d_9 7.00 min
Ritonavir 7.80 min
Etravirine 8.60 min

Calcul de la concentration intracellulaire « vraie » (patients) :

$$\text{Conc. intracell.} = \frac{\text{Conc. mesurée} * \text{Vol. dilution}}{\text{Vol. cellule} * \text{Qté cellule}} \qquad \text{ex} : \frac{3.5\ \text{ng/mL} * 0.2\ \text{mL}}{0.4\text{x}10^{-9} * 12\text{x}10^{6}} = 145.8\ \text{ng/mL}$$

Où :

Conc. intracell = concentration intracellulaire finale en tenant compte du nombre de cellules et de leur volume [ng/mL]
Conc. mesurée = concentration mesurée par LC-MS/MS [ng/mL]
Vol. dilution = volume de dilution du culot cellulaire [mL]
Vol. cellule = volume d'une cellule, en général 0.4 pL = $0.4\text{x}10^{-9}$ mL
Qté cellule = quantité de cellules (10^6 cell.) dans le culot de patients (cf. comptage cellulaire)

Etiquette avec nom patient

CENTRE HOSPITALIER UNIVERSITAIRE VAUDOIS
DEPARTEMENT DE MEDECINE
SERVICE DES MALADIES INFECTIEUSES
et DIVISION DE PHARMACOLOGIE CLINIQUE

Etude pharmacocinétique du Raltegravir intra-cellulaire et plasmatique chez les patients infectés par le VIH

DOCUMENT DE RECOLTE DES INFORMATIONS

Date actuelle : .....
N° patient : .....
Nom : .....
Prénom : .....
Date de naissance : .....
Consentement donné le :.....

Etiquette patient

Caractéristiques démographiques

Sexe : M ☐ F ☐

Race : caucasien ☐ africain ☐ asiatique ☐ métis ☐

Mensurations : poids : ..... kg taille : ..... cm

Critères d'inclusion:

	oui	non
• Patient(e) traité depuis plus de 21 jours par une HAART comprenant du raltégravir (Isentress®) 2 x 400 mg/j.	☐	☐
• Test de grossesse négatif pour les femmes susceptibles de procréer	☐	☐

Critères d'exclusion

• Femmes enceintes ou qui allaitent	☐	☐

Etiquette avec nom patient

CENTRE HOSPITALIER UNIVERSITAIRE VAUDOIS
DEPARTEMENT DE MEDECINE
SERVICE DES MALADIES INFECTIEUSES
et DIVISION DE PHARMACOLOGIE CLINIQUE

1. Renseignements médicaux

Evaluation clinique

Insuffisance : cardiaque ☐ hépatique ☐ rénale ☐ malabsorption ☐

Remarques :.....
.....

Adhésion : "Combien de doses avez-vous oublié lors des dernières 4 semaines ?"

Question posé par l'infirmière le jour de la pharmacocinétique :.....

Traitement antirétroviral actuel

Médicament (Spécialité)	**N° lot**	**Dose** [mg]	**Nombre de prise par jour**	**Heures des prises**	**Moment des prises** (av,pd,ap. repas)	**Date introduction** (j/m/a)
			……x/jour	……h……min		……/……/……
			……x/jour	……h……min		……/……/……
			……x/jour	……h……min		……/……/……
			……x/jour	……h……min		……/……/……
			……x/jour	……h……min		……/……/……
			……x/jour	……h……min		……/……/……

Autres médicaments actuels

Médicament (Spécialité)	**Dose** [mg]	**Nombre de prise par jour**	**Heures des prises**	**Date d'introduction**
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……
		……x/jour	……h……min	……/……/……

Etiquette avec nom patient

CENTRE HOSPITALIER UNIVERSITAIRE VAUDOIS
DEPARTEMENT DE MEDECINE
SERVICE DES MALADIES INFECTIEUSES
et DIVISION DE PHARMACOLOGIE CLINIQUE

2. Renseignements concernant le prélèvement

Inscrire les 3 dernières doses du Raltégravir

Médicament	Date de prise	Heure de prise	Moment de la prise (av, pd, après repas)	Heure du repas	Composition du repas
Raltégravir					
Raltégravir					
Raltégravir					

Concentrations plasmatiques et cellulaires

Temps [h]	Heure prise de sang prévue [h]	réelle [h]	Prélèvements sanguins à effectuer [ml] tot	type	Commentaires
Pré-dose (0)	7h30		18.5	EDTA + *CPT* chimie + hémato	
administration de la dose	8h00		(pas d'échant.)		
1	9h00		5.5	EDTA	
3	11h00		13.5	EDTA + *CPT*	
6	14h00		5.5	EDTA	
8	16h00		13.5	EDTA + *CPT*	
12	20h00		13.5	EDTA + *CPT*	

Heure de prise des autres traitements antirétroviraux

Médicament (Spécialité)	Dose [mg]	Nombre de prise par jour	Date et heure de la dernière prise
		……x/jour	……/……/2008 à ……h……min
		……x/jour	……/……/2008 à ……h……min
		……x/jour	……/……/2008 à ……h……min
		……x/jour	……/……/2008 à ……h……min
		……x/jour	……/……/2008 à ……h……min

Heures de prise du dernier repas standardisé : Début :……
Fin :……

3. Résultats de laboratoire (date:…/…/…)

(pour les résultats de la chimie et de l'hématologie, voir *Molis*)

Virémie *(copies/mL) :……*
CD4 *(cell/mm^3) :……(=….%)*

Test de grossesse *(urine) :……*