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Quantification of darunavir and etravirine in human peripheral blood mononuclear cells using high performance liquid chromatography tandem mass spectrometry (LC–MS/MS), clinical application in a cohort of 110 HIV-1 infected patients and evidence of a potential drug–drug interaction

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ABSTRACT

Objectives: To describe the validation of a sensitive high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method allowing the simultaneous quantification of darunavir (DRV) and etravirine (ETR) in peripheral blood mononuclear cells (PBMCs) and its application in a cohort of HIV-1 infected patients.

Methods: Blood samples were obtained from 110 patients. PBMCs were isolated using density gradient centrifugation. Drug extraction from PBMCs was performed with a 60:40 methanol–water (MeOH–H₂O) solution containing deuterated IS (DRV-d9 and ETR-d8). The chromatographic separation was performed on a RP18 XBridge™ column.

Results: The geometric mean (GM) of cell associated concentration ([DRV]_{CC}) and plasmatic concentration ([DRV]_{plasma}) were 360.5 ng/mL (CI95%:294.5–441.2) and 1733 ng/mL (CI95%:1486–2021), respectively. A geometric mean intracellular (IC)/plasma ratio (GMR) of 0.21 (CI95%:0.18–0.24) was calculated. Adjusted for dose/body surface area and post-intake time, a statistically significant correlation was observed between [DRV]_{plasma} and the eGFR ($p = 0.002$) and between [DRV]_{plasma} and the concomitant use of ETR ($p = 0.038$). For the 10 patients receiving ETR in addition to DRV, the GM of [ETR]_{plasma} (available for 8 out of 10 patients) and [ETR]_{CC} were 492.3 ng/mL and 2951 ng/mL respectively. The GMR of ETR was 7.6 (CI95%: 3.61–13.83).

Conclusions: A handy and sensitive high performance LC–MS/MS method allowing the simultaneous quantification of DRV and ETR in PBMCs has been described and successfully applied in the largest cohort of DRV-treated patients reported to date. ETR accumulates more efficiently in PBMCs compared to DRV. We have also highlighted a possible impact of ETR on DRV plasma concentrations requiring further investigations.

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1. Introduction

Darunavir (Prezista® DRV) is a potent protease inhibitor (PI) used as a component of highly active antiretroviral therapy (HAART) in combination with the pharmacokinetic (PK) booster ritonavir for the treatment of Human Immunodeficiency Virus-1 (HIV-1) infected patients [1,2]. Etravirine (Intelence® ETR) is another anti-HIV-1 drug belonging

to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class employed for the management of HIV-1 infections in the presence of viral mutations associated with NNRTI resistance [3].

Therapeutic drug monitoring (TDM) of antiretrovirals (ARV) based on drug plasma concentrations is currently widely available to control anti-HIV therapy [4–6]. This reactive strategy allows clinician to better manage the risk of drug under- and over-exposure. Nevertheless, while drug overshoot is thought to be directly linked to the occurrence and the severity of adverse drug reactions (ADRs) [5], it is not clear whether drug plasma level is an indicator of its effectiveness. Indeed, the targets of PIs and NNRTIs are viral proteins located within the infected cells. Thus, at least intuitively, it appears that intracellular (IC) concentrations of ARV drugs should better correlate with their efficacy

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than plasma concentrations. Therefore, quantitative determination of DRV and ETR in human peripheral blood mononuclear cells (PBMCs) may more adequately reflect treatment effectiveness than quantification of these drugs in plasma [7–12].

Previously, only few methods have been developed for the quantification of both antiretroviral drugs in PBMCs [13,14]. The present paper describes the development and the full validation of a handy and sensitive high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method allowing the simultaneous quantification of DRV and ETR in PBMCs and its clinical application in a cohort of 110 naïve and experienced HIV-1 infected patients.

2. Material and methods

2.1. Study design

HIV-1 infected patients were recruited from the AIDS Reference Centre of the Cliniques Universitaires Saint-Luc (Brussels, Belgium) between November 2012 and February 2014. Patients of 18 years and older with confirmed HIV-infection and treated by DRV with/without ETR for at least one month prior to inclusion were eligible for the study.

In addition to the samples routinely collected for the clinical follow-up of the patient (viral load, CD4 cell count), two additional samples used for determination of trough plasma and IC concentrations were drawn immediately before next drug intake, with the highest timing precision conceivable given the ambulatory context of the study recruitment. In order to obtain a post-intake delay as close as possible to the trough concentration sampling time, each patient was contacted by phone a few days before the day of the visit for the study to be asked not to take the medication prior to blood sampling.

The protocol (NCT02514369) was approved by the local ethical committee (B403201214460) and a written informed consent was obtained from each patient taking part to the study.

2.2. Chemicals and reagents

DRV, ETR, and their respective deuterated internal standards (IS) DRV-d9 and ETR-d8 were purchased from Toronto Research Chemicals (Ontario, Canada); methanol HPLC grade from Biosolve (Dieuze, France); formic acid from Sigma-Aldrich (Seelze, Germany) and the Dulbecco's phosphate buffered saline (DPBS) solution from Invitrogen (Life Technologies, Gent, Belgium). HPLC grade water was produced with Milli-Q water purification system by Millipore (Darmstadt, Germany). Blank PBMCs used for calibration curves and quality control (QC) samples were isolated from the blood of healthy volunteers. PBMCs count was performed on a Sysmex K-1000 haematology analyzer (Norderstedt, Germany).

2.3. LC–MS/MS system and chromatographic conditions

DRV and ETR measurements were performed by LC–MS/MS, using a Quattro micro tandem-mass spectrometer (Micromass UK, Manchester, UK) fitted with a Z-spray ion source. The instrument was directly coupled to a Waters 2795 Alliance (high throughput) HT LC system, with an integrated auto sampler thermostated at 10 °C (Waters, Milford, USA). The chromatographic separation was performed on RP18 2.5 µm column (2.1 × 75 mm) (Waters, Milford, MA, USA) maintained at 35 °C. The 20 µL injected aliquot was eluted at a mobile phase flow rate of 0.3 mL/min. The mobile phase consisted of water (eluent A) and methanol (eluent B), both containing 10 mM formic acid. Initial eluent composition was 90% A directly followed by a linear 1.0 min ramp to 10% A, which was maintained for 4.49 min. The mobile phase returned to 90% A at 5.5 min. Equilibration time was 1 min. Ionization was in the positive ion mode using the following settings: capillary voltage 3.5 kV, cone voltage 22/22/54/54 V (DRV/DRV-d9/ETR/ETR-d8),

source temperature 125 °C, desolvation temperature 300 °C at a nitrogen flow of approximately 650 L/h, and collision gas (high-purity argon) pressure $3 \cdot 10^{-3}$ bar. DRV, DRV-d9, ETR, and ETR-d8 were monitored in multiple reaction monitoring mode (MRM) by detecting specific product ions: DRV m/z 548.24 > 113.2, DRV-d9 m/z 557.36 > 401.26, ETR m/z 435.09 > 144.4, ETR-d8 m/z 443.18 > 304.33. Collision energies were 21/15/49/45 for DRV/DRV-d9/ETR/ETR-d8 respectively.

2.4. Collection of PBMCs from patients

About 8 mL of blood was collected in cell preparation tube (CPT) from Becton Dickinson Vacutainer® systems. Blood samples were first centrifuged at $1850 \times g$ for 15 min at room temperature (RT). The cell layer was collected with a Pasteur pipette and transferred to a 15 mL size conical centrifuge tube. The volume was completed to 15 mL with cold DPBS to block enzymatic activity and to avoid active transport out of the cells [15]. PBMCs were then washed 2 times according to the manufacturer's instructions (at $650 \times g$ for 10 min at 4 °C). The cell pellet was resuspended in 1 mL of DPBS. 20 µL aliquot was diluted in 180 µL of DPBS and directly used for cell counting using a Sysmex analyzer. After a final centrifugation of the remaining 980 µL at $650 \times g$ (10 min at 4 °C), the supernatant was aspirated and the cell pellet was stored immediately at –80 °C until the day of drug extraction. This method was adapted from Elens et al. [8].

2.5. Preparation of blank PBMCs

Approximately 8 mL of blood from healthy volunteers was collected in heparin tubes (Sarstedt Monovette) and was transferred in LeucoSep™ tubes (Greiner Bio-One, Kremsmünster, Austria). Blood samples were centrifuged at $800 \times g$ for 20 min according to the manufacturer's instructions. The thin mononuclear layer was collected and successively washed twice with DPBS (at $250 \times g$ for 10 min). The final dry cell pellet was stored at –20 °C.

2.6. Stock solutions, calibrators and quality controls

DRV and DRV-d9 stock solutions were obtained by diluting the compounds in methanol to reach a final concentration of 0.5 mg/mL and 1 mg/mL, respectively. ETR and ETR-d8 were diluted in chloroform to obtain a concentration of 1 mg/mL. All stock solutions were then kept at –20 °C. A working solution of DRV and ETR at a concentration of 1250 ng/mL was prepared in 60:40 MeOH:H₂O. Similarly, a solution of DRV-d9 and ETR-d8 at a concentration of 4000 ng/mL was also prepared.

DRV and ETR working solutions were diluted in 60:40 MeOH:H₂O to obtain 6 calibrators (1.25, 6.25, 12.5, 62.5, 93.75, 125 ng/mL) and 3 QC solutions (2.5, 25, 75 ng/mL) containing all DRV-d9 and ETR-d8 at a fixed concentration of 20 ng/mL.

2.7. Sample processing

Blank PBMCs were thawed at RT and resuspended in 400 µL of the calibrators or QC diluted in the extraction solution (60:40 MeOH:H₂O solution containing DRV-d9 and ETR-d8 at 20 ng/mL). PBMCs from HIV-infected patients were resuspended with 400 µL of the cell extraction solution. After vortex mixing (10 s), the resuspended pellets were sonicated for 5 min and subsequently placed on a horizontal shaker at 250 rpm for 6 h. The mixture was finally centrifuged ($10,500 \times g$, 10 min) and the supernatant was transferred to an automatic autosampler vial.

2.8. Analytical method validation

The assay was fully validated according to the U.S. Food and Drug Administration (FDA) [16].

2.8.1. Linearity

Five calibration curves on three separate days were run during the validation procedure. Each calibration curve was obtained using the six calibrators and a blank control (1.25, 6.25, 12.5, 62.5, 93.75, 125 ng/ml).

2.8.2. Precision, accuracy, lower limit of quantification and limit of detection

Accuracy and precision of the method were determined through the evaluation of five replicates at the three QC concentrations on three consecutive validation days ($n = 15$). Accuracy was expressed as the bias (% deviation) from nominal concentration while precision was expressed as the coefficient of variation in % (CV). Within and between days precision (% CV) at each concentration level should not be greater than 15%. Similarly, intra- and inter-day accuracy should be within two standard deviations from the nominal concentration.

Lower limit of quantification (LLOQ) was estimated by quantifying blank PBMCs spiked with decreasing concentrations of DRV and ETV and was defined as the lowest concentration, of 10 replicates, with an accuracy positioned between 80% and 120% of the nominal concentration with a CV lower than 15%. Limit of detection (LOD) was estimated as the lowest concentration giving chromatographic peaks with a signal-to-noise ratio > 3 .

2.8.3. Extraction recovery

Extraction recovery was evaluated in five replicates at two concentrations by comparing the mean peak area responses of blank PBMCs spiked at two concentrations (5 ng/ml, 100 ng/ml) to those of a pure spiked solution (60:40 MeOH:H₂O) representing 100% recovery. In order to meet acceptance criteria, the extraction recovery should be between 80 and 120% and must be quite stable.

2.8.4. Specificity and selectivity

The selectivity was assessed by analysing a set of 15 different blank PBMCs.

2.8.5. Matrix effect

The post-extraction addition technique has been used to estimate matrix effect [17]. For both DRV and ETV, matrix effect was determined at the three QC levels and LLOQ concentrations (2.5, 25, 75 and 1.25 ng/mL) in 6 replicates, using 6 different-drug-free PBMCs samples (obtained from different healthy individuals) for each aliquot.

2.8.6. Determination of cell associated concentration: $[DRV]_{cc}$ and $[ETV]_{cc}$

Based on the calibration curves, drug concentrations in the cell extracts were first expressed in ng/ml. The absolute amount of DRV and ETV (ng) in PBMCs was obtained by multiplying the drug concentration by 0.4 (400 μ L of extraction solution). This amount was then divided by the number of cells present in the cell extract (in millions) and the PBMC concentration (also in ng/ml) was finally calculated assuming a mean PBMC volume of 0.4 pL [8,11].

2.9. Determination of plasma drug concentration: $[DRV]_{plasma}$ and $[ETV]_{plasma}$

For each patient, blood samples dedicated to plasma drug quantification were obtained on heparinised tubes collected simultaneously with the CPT Vacutainer® tubes. They were immediately centrifuged at $1125 \times g$ for 10 min at RT. Plasma was then collected and stored at $-20^\circ C$ until the day of quantification. Concentrations of DRV in plasma ($[DRV]_{plasma}$) and ETV in plasma ($[ETV]_{plasma}$) were determined using a ultra high-pressure liquid chromatography (UPLC) with diode array detection (DAD) [18] that is presently routinely used in our laboratory. Using this validated method, the laboratory has obtained successful results in the external quality assessment scheme organized by SKML (The Netherlands) on antiretroviral drugs including DRV and ETV.

2.10. Statistical analysis

Statistical analysis was performed using SPSS Software package version 22 for Mac (SPSS Inc., Chicago, IL, USA). DRV and ETV plasma and IC concentrations were log-transformed for normalisation of the distribution. Descriptive statistics were then calculated and are reported as inverse log values. To assess the possible influence of categorical variables such as ethnicity, gender, or concomitant use of other antiretroviral drugs on different PK parameters, Student t-test (2 groups) or one-way ANOVA (> 2 groups) were used.

Linear regressions were performed to assess the impact of each continuous variable such as age, post-intake delay, body mass index and renal function (evaluated by estimated glomerular filtration rate (eGFR) assessed by the Modification of Diet in Renal Disease (MDRD)) on PK parameters. Multivariate linear regressions were performed to estimate the influence of these variables adjusted for individual doses of the drugs reported on body surface area and for post-intake time.

Finally, $[DRV]_{plasma}$ and $[DRV]_{cc}$ were studied simultaneously in generalized estimating equation (GEE). Regressions were computed by quasi-least-squares, estimating the correlation between the two dependent variables according to Chaganty and Shults [19]. Significance was estimated by chi-score on robust sandwich variance [20].

All tests were considered as statistically significant for bilateral p -values ≤ 0.05 .

Based on the link between $[DRV]_{plasma}$ and different post-intake times, an estimated half-life ($T_{1/2}$) was calculated by linear regression of the natural logarithm of the individual DRV concentrations against the post-intake times. This estimation has the drawback of not having successive points for the same patient.

3. Results

3.1. Validation results

Typical chromatograms of DRV and ETV samples at LLOQ are shown in Fig. 1.

The assay was linear over the tested concentration range of 1.25–125 ng/ml for DRV and ETV. The equations of the lines for each drug were $y = 0.040x - 0.007$ and $y = 0.070x - 0.035$ for DRV and ETV, respectively with correlation coefficients (r^2) always greater than 0.99 ($p < 0.0001$).

The method was accurate and precise with respectively bias always lower than two standard deviations from the nominal concentration and CV ranging from 4.6 to 14.3% (Table 1). The LOD and LLOQ values were 0.60 and 1.25 ng/ml for DRV and 0.62 and 1.25 ng/ml for ETV.

All recovered concentrations were within 80–120% of nominal concentrations (5 and 100 ng/ml) and showed a good reproducibility (CV ranging from 2.5 to 10.7%).

Blank PBMCs did not show any interference for the specified ions detected.

At the three QC, matrix effect was acceptable, with ion suppression $< 10\%$ for DRV, ETV and internal standard (Table 1).

3.2. Clinical application of the method

In total, 110 HIV-positive patients participated to the study. All quantification analyses have been performed on the same day to minimize the variability of the results.

4 patients were excluded because of a suspicion of a noncompliance in the presence of an uncontrolled viral replication ($[DRV]_{cc}$ and $[DRV]_{plasma}$ were $< LOD$). Two other patients had a $[DRV]_{cc} < LOD$ solely and were then excluded.

67.3 % of the patients were treatment-naïve receiving DRV boosted by ritonavir (DRV/r) 800 mg/100 mg QD while 30.7% of the population consists of treatment-experienced patients receiving DRV/r 600 mg/100 mg BID. Two patients had unusual posology (DRV/r 600 mg/

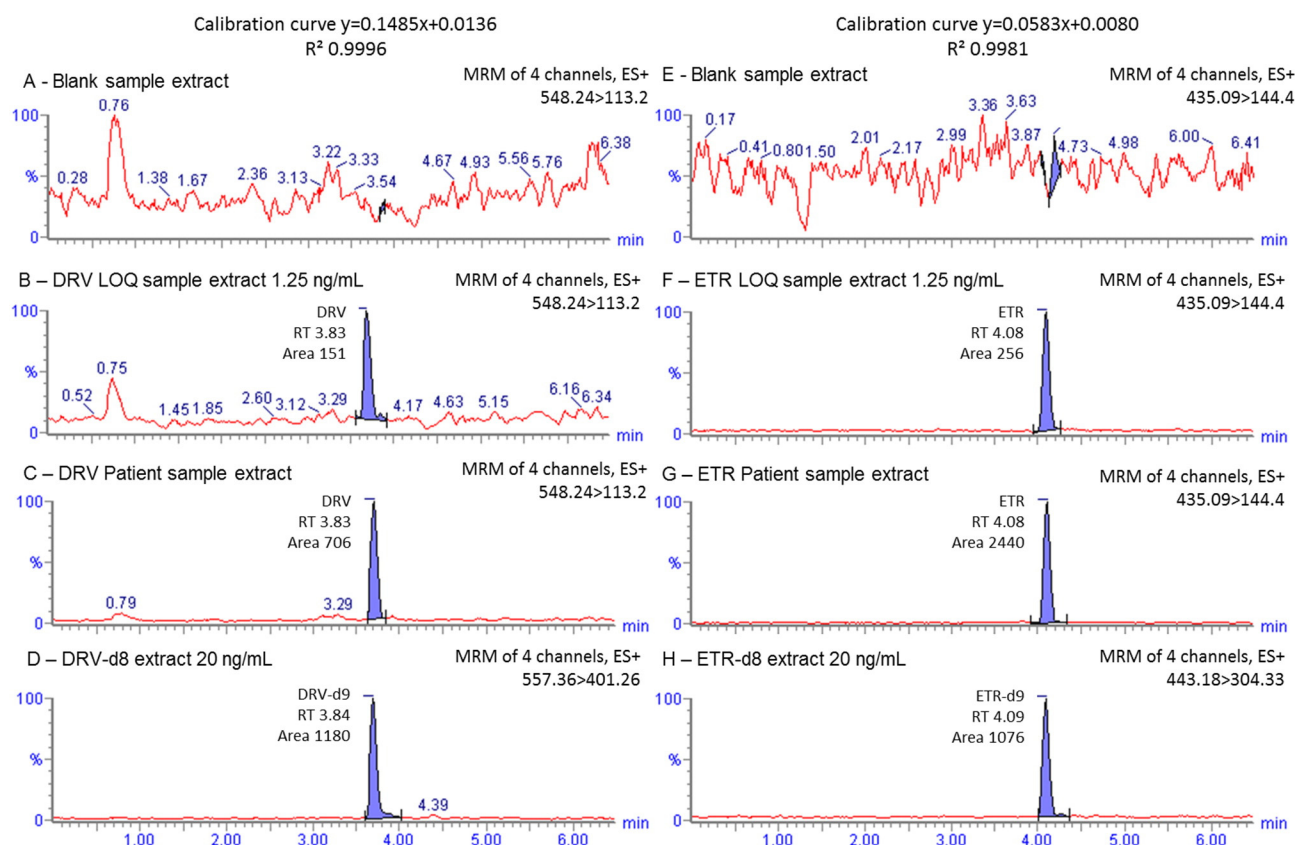


Fig. 1. DRV and ETR typical chromatograms of PBMC extract obtained either with a blank sample (A, E) and internal standard (D, H) or with a LLOQ sample (B, F). In addition, two patient ion chromatograms of PBMC extract (C–G) are reported. In these examples, DRV and ETR concentration was 10.4 ng/mL and 34.2 ng/mL (before transformation to obtain IC concentrations), respectively. RT: retention time; ES+: electrospray positive mode; MRM of 4 channels: multiple reaction monitoring of 4 different ion transitions, Y axis express as relative intensity (%).

100 mg and 900 mg/100 mg, respectively). The general characteristics of the patients are listed in Table 2.

The geometric mean of $[DRV]_{CC}$ and $[DRV]_{plasma}$ was 360.5 ng/ml (CI95% 294.5–441.2 ng/ml) and 1733 ng/ml (CI95% 1486–2021 ng/ml), respectively. The geometric mean of $[DRV]_{CC}$ was 342.5 ng/ml and 518.1 ng/ml for the patients receiving DRV/r QD versus BID respectively ($p = 0.083$).

A good correlation between cell-associated and plasmatic concentrations was observed ($r^2 = 0.501$, $b = 0.930$ p -value < 0.0001 , Fig. 2A), i.e. about 50% of the variability of the DRV cell-associated concentration could be explained by the plasma concentrations. A geometric mean IC/plasma ratio (GMR) of 0.21 (CI95% 0.18–0.24) was calculated.

In univariate analysis, there was no statistically significant correlation between age, gender, ethnicity, weight or concomitant use of other antiretroviral drugs and $[DRV]_{plasma}$ (Supplemental data Table 1). A statistically significant relationship between the eGFR and $[DRV]_{plasma}$

was observed ($r^2 = 0.058$ $p = 0.014$, Supplemental data Table 1, Fig. 3). In the model, it appeared that $[DRV]_{plasma}$ increases by a factor of 1666 when GFR decreases by 50%.

Adjusted for dose/body surface area and post-intake time by multivariate regression, a statistically significant correlation was observed between $[DRV]_{plasma}$ and the eGFR ($r^2 = 0.329$ $p = 0.002$, Supplemental data Table 1) and between $[DRV]_{plasma}$ and the concomitant use of ETR. ETR co-administration multiplies the $[DRV]_{plasma}$ by a factor of 0.51 ($r^2 = 0.321$ $p = 0.038$, Supplemental data Table 1).

The $T_{1/2}$ was calculated as 10.75 h (CI95% 8.01–16.33) for the $[DRV]_{plasma}$ and 12.91 h (CI95% 8.12–31.59) for the $[DRV]_{CC}$. After exclusion of the 10 patients with ETR, the $T_{1/2}$ became 10.92 (CI95% 8.14–16.56) and 13.25 (CI95% 8.31–32.77) h for $[DRV]_{plasma}$ and $[DRV]_{CC}$, respectively. Correlation between post-intake time and $[DRV]_{plasma}$ for patients with or without ETR is shown in Fig. 4. The $T_{1/2}$ for the 10 patients with ETR was calculated as 3.33 h (CI95% 2.31–5.99) and 2.03 h (CI95% 1.52–3.09) for $[DRV]_{plasma}$ and $[DRV]_{CC}$, respectively. These differences in the plasma and IC $T_{1/2}$ with or without concomitant use of ETR are statistically significant ($p = 0.006$ and 0.002 for $[DRV]_{plasma}$ and $[DRV]_{CC}$, respectively).

Consistently, GEE for simultaneous analysis of $[DRV]_{plasma}$ and $[DRV]_{CC}$ adjusted for dose/body surface area and post-intake time confirms they are significantly correlated with the following factors:

- concomitant use of ETR ($p = 0.038$)
- eGFR ($p = 0.005$).

The ratio IC/plasma of DRV shows no significant interaction with the factors studied.

For the 10 patients receiving concomitant ETR, the geometric mean of $[ETR]_{plasma}$ (available for 8 out of 10 patients) was 542.6 ng/ml

Table 1

Accuracy (% deviation from nominal concentration), intra- and interassay precision (CV in %) and matrix effect. (n = 5 in three separate runs).

Anti-HIV drug	Nominal Concentration (ng/ml)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)	Matrix effect (%)
DRV	2.5	6.1	9.8	11.5	-8.1
	25	3.6	4.7	5.8	-5.3
	75	3.1	3.5	4.9	-7.8
ETV	2.5	3.2	12.1	14.4	-9.1
	25	5.6	6	6	-8.3
	75	6.2	4.6	4.6	-9.6

Table 2
Patient characteristics at day of sampling.

	Darunavir Prezista®
Number of patients in total	104
Number of patients according posology per day	600 mg (n = 1) 800 mg (n = 70) 900 mg (= 1) 1200 mg (= 32)
NNRTI	ETR (n = 10) NVP (n = 2) NNRTI-free (n = 92)
NRTI	ABC/3TC (n = 15), FTC/TDF (= 56), 3TC (n = 13), TDF 245 mg (n = 3), NRTI-free (n = 17)
RAL	n = 29
MVC	n = 10
Age (mean ± SD)	47.9 ± 12.1 years
Body Mass Index (mean ± SD)	25.45 ± 4.89
Gender	Male = 68 (65.4%) Female = 36 (34.6%)
Ethnic origin	Caucasian: 62 (59.6%) African: 39 (37.5%) Asian: 2 (1.9%) South American: 1 (1%)
CD4 cell count (cells/mm ³) median [min–max–IQR]	532 [33–1641–407]
Nadir (cells/mm ³) median [min–max–IQR]	144 [2–671–146]
HIV-1 RNA < 37 cps/ml: number of patients (%)	78 (75%)
HIV-1 RNA > 37 cps/ml: number of patients (%)	26 (25%)
Median [min–max–IQR] cps/ml	104 [41–64460–168]
Duration of treatment (mean ± SD)	21.8 ± 15, 2 months
GFR mean ± SD (MDRD)	86.97 ± 23.35 mL/min/1.73 m ²

NNRTI: non-nucleoside reverse transcriptase inhibitors, NRTI: nucleoside reverse transcriptase inhibitors, RAL: raltegravir, MVC: maraviroc ETR: etravirine, NVP: nevirapine, ABC: abacavir, 3TC: lamivudine, FTC: emtricitabine, TDF: tenofovir disoproxil fumarate. NNRTI-free: without any NNRTI. NRTI-free: without any NRTI.

(CI95% 326.9–900.7 ng/ml). The geometric mean of [ETR]_{cc} was 3018 ng/ml (CI95% 1339–6800 ng/ml) but extremely variable compared to plasma levels (CV = 124.2 versus 42.3%, respectively). The GMR was 7.06 (CI95% 3.61–13.83). There was a borderline significant correlation between log[ETR]_{cc} and log[ETR]_{plasma} ($r^2 = 0.566$, $b = 1.43$, $p = 0.031$) but this relatively high p-value might be the consequence of the low number of data for ETR (Fig. 2B). The slope value was >1, probably reflecting that ETR accumulates more in the cells than DRV. Given the low number of patients receiving ETR, it was not possible to analyse the factors influencing its PK.

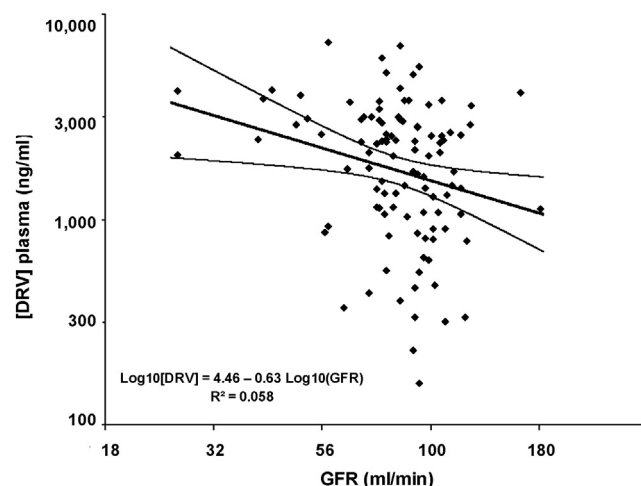


Fig. 3. Effect of the eGFR on the [DRV]_{plasma}. Lines represent the average trend ± SD.

4. Discussion

The first aim of our study was to develop and validate a sensitive, accurate and precise LC–MS/MS method for the simultaneous quantification of DRV and ETV in PBMCs. The use of deuterated IS allowed us to validate our method over a range of 1.25–125 ng/ml in PBMCs matrix (drug concentrations in the cell extracts before transformation in IC concentration) for DRV and ETR with excellent correlation coefficients ($r^2 > 0.99$). Our method was proven to be accurate, precise and specific at three different concentrations (low, medium and high) reflecting almost all scenarios in the clinical setting. The previous reported methods used for DRV [11,21,22] or both DRV/ETR IC quantification [13,14] did not use deuterated drugs as IS. D'Avolio et al. were able to quantify 14 ARV in a unique chromatographic run [13]. However, their method was based on a simple LC–MS detector which reduces the specificity of the quantification method compared to a tandem mass spectrometry detector [13]. Moreover, they used Quinoxaline as unique IS for the 14 drugs which had not the same retention time than either DRV or ETR (8.6 min for Quinoxaline versus 10.2 and 15.2 min for DRV and ETR, respectively) and can thus not correct the signal for detection variability. Additionally, the running time was reduced to 6.5 min versus 25 min in their method. This is a considerable gain of time when analysis of large batches of samples is performed, which situation is very frequent in clinical and research studies. Both methods are quite similar in terms of precision and accuracy. However, the sensitivity of their technique

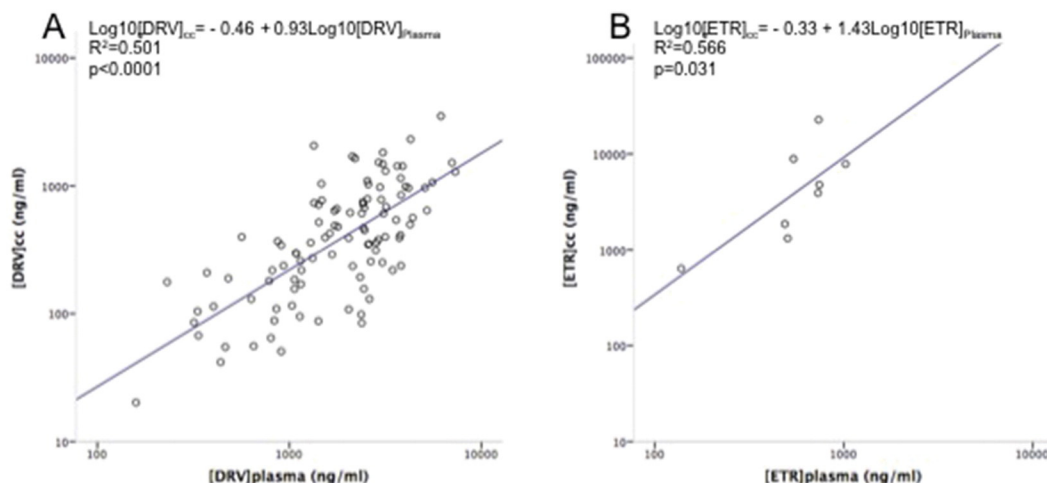


Fig. 2. Relationship between the IC and the plasma concentrations for DRV (A) and ETR (B), respectively, expressed in ng/ml.

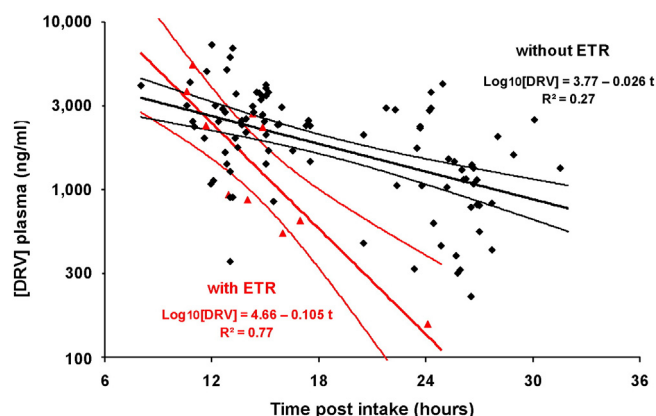


Fig. 4. Correlation between post intake time and $[DRV]_{\text{plasma}}$ for patients with ETR (in red) or without (in black). Lines represent the average trend $\pm$ SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seems to be 10-fold better than ours. However, the sensitivity of our method seems sufficient to be applied in clinical setting (see clinical application) and the loss of sensitivity is counterbalanced by a higher specificity of LC–MS/MS compared to LC–MS. Recently, another method using a fluorescence detector has been reported but only for DRV IC quantification [21]. Even if probably more available in routine labs, this detection system is less specific than tandem mass spectrometry. Moreover, its sensitivity appeared substantially lower than the present method with a linearity range of 5–100 ng/10⁶ cells. Finally, their technique only allows DRV quantification and not ETR while both drugs are frequently co-administrated in HIV-1 experienced patients. Furthermore, this drug combination is characterized by huge PK variations and both drugs can be victim and/or perpetrate PK interactions (see below).

In a second step, the objective was to demonstrate that the method could be easily applied to clinical situations by quantifying DRV and ETR in a large cohort of 110 patients with 10 patients receiving both drugs in combination. As expected and consistent with previous studies [23] DRV concentrations in PBMCs and in plasma were extremely variable with CV of 99.4 and 66.0 % respectively but noticeably higher than the LLOQ, confirming that the sensitivity of our technique was good enough for its use in practice. Interestingly, a consistent correlation between $\log[DRV]_{\text{plasma}}$ and $\log[DRV]_{\text{cc}}$ was observed with a slope of 0.93 (Fig. 2A). This probably indicates that DRV does not accumulate within the cell as, for a large increase in plasma concentration, only a moderately higher IC concentration is observed. Drug accumulation in cell is not only a passive process but also includes dynamic transport. The magnitude of cell accumulation clearly depends on multiple concomitant mechanisms chiefly plasma protein binding, drug lipophilicity or ionization and influx/efflux transmembrane transport activity. DRV has been identified as an ABCB1, OATP1A2 and ABCC2 substrate and their activity could potentially modulate DRV passage into the cells expressing those proteins and thus the relationship between plasma and IC concentrations.

Despite similar $[DRV]_{\text{plasma}}$, the $[DRV]_{\text{cc}}$ reported here are lower than those described in other studies [13,14]. In their study, Ter Heine et al. found in cells C_{trough} of 1450 $\pm$ 1110 ng/ml (mean $\pm$ SD, $n = 10$) [14]. Consequently, the accumulation ratio observed was higher in their cohort than in the present study, with values of 1.32 versus 0.21. However, that study involved only 10 experienced patients receiving 600 mg/100 mg DRV/r BID while in our study, about 70% of patients were naïve-treatment patients receiving 800 mg/100 mg DRV/r QD. Interestingly, it has been recently shown that RTV dosing significantly affects the extent of DRV penetration into the cerebrospinal fluid [24]. Indeed patients receiving DRV/r QD showed not only lower CSF DRV C_{trough} but also lower CSF-to-plasma ratios (0.32% versus 0.90%),

suggesting that the penetration of the drug across the blood-brain-barrier (BBB) (where ABCB1 is expressed) is lower in the once-daily regimen than in the twice-daily regimen. This could be the consequence of a dose-dependent RTV inhibition of transporters, suggesting possibly that ABCB1 (also expressed in PBMCs [24,25]) is less inhibited with lower doses of RTV and thus allows lower cell accumulation in the case of a once-daily administration. This is in accordance with the fact that the majority of our patients were naïve and thus treated with lower doses of RTV. The second study that investigated the DRV IC PK was reported by Jackson et al. [11]. In this study, the DRV IC AUC-to-plasma ratio ranged between 4.9 and 5.6. In their cohort, all patients received 800 mg/100 mg DRV/r QD in association with raltegravir (RAL) 800 mg in once or twice daily. In our cohort, only approximately 30% of our patients were given RAL on a BID basis. Even if it is difficult to decipher the neat impact of these study design differences, we cannot exclude that part of the discrepancies observed are due to differences in treatment regimens. Another very conceivable explanation is certainly methodological. Indeed, in their review, Bazzoli et al. summarized findings from studies on IC concentrations of antiretrovirals and highlighted clear limitations, e.g. small patient numbers, poor study design and methodological differences [7]. The differences between our IC concentration values and those reported by Jackson [11] probably also come from their analytical procedure and more precisely from the cell isolation step using a single cycle of cell washing to remove drug contamination from plasma. In our method, 3 ice-cold washing steps of the cell pellets are included as described in Elens et al. [8]. In this last study, accumulation ratios were in the same range as in our study with 0.42 for Lopinavir (LPV/r) and 0.18 for Tipranavir (TPV/r). Jones et al. have demonstrated a hierarchy in the in vitro IC accumulation of PIs with: nelfinavir (NFV) > saquinavir (SQV) > zidovudine (ZDV) > indinavir (IDV) [26]. Later, Khoo et al. have confirmed this tendency with accumulation ratios of 4.01 for SQV/r and 0.29 for IDV/r, using a three-step washing methodology [27]. In a recent conference report, D. Back et al. (Liverpool) highlighted that the unique washing step methodology potentially over-estimates IC concentration by inclusion of residual plasma drug contamination and drug bound to cell membrane. Furthermore, it has been shown in a previous report that, following 60 min of incubation of EFV-loaded PBMCs in a drug-free medium at 4 °C, approximately 97% EFV remained cell-associated compared to about 70% at 37 °C, reflecting that working at 4 °C limits analyte-loss during these additional washing steps [15]. In conclusion, using quite similar methodologies, we can place DRV/r in the same range of LPV/r, TPV/r and IDV/r in the hierarchy of IC accumulation much lower than ETR.

Drug–drug interactions between antiretroviral drugs are complex and remain a challenge when starting a treatment. It has been reported that ETR is an inducer of CYP3A and a weak inhibitor of CYP2C9, CYP2C19 and ABCB1 Scholler-Gyure, Kakuda [28]. As DRV is primarily metabolized by CYP3A and is a substrate of multiple ABC, including ABCB1, ETR has the potential to modulate the DRV PK pathway by inducing CYP3A and/or inhibiting ABCB1.

This kind of interaction was demonstrated for other PIs such as atazanavir (ATV) and fosamprenavir (FPV) which are also metabolized by CYP3A. In an open-label crossover study in 32 healthy volunteers, it was shown that ETR co-administrated with unboosted ATV (400 mg QD) or boosted ATV/r (300/100 mg) reduced ATV AUC_{24h} by 17% and 14% respectively possibly because of induction of CYP3A by ETR [29]. In the same study, co-administration of ETR with ATV or ATV/r decreased ATV C_{trough} by 47% and 38% respectively. FPV is the phosphate ester prodrug of the PI amprenavir (APV). APV is the PI with the closest structural resemblance to DRV [30] and is also a CYP3A/ABCB1 substrate [31]. In a small cohort of 8 HIV-1 positive patients receiving boosted FPV/r 700/100 mg BID, $[APV]_{\text{plasma}}$ and AUC_{12h-Cmin} were significantly increased by 69% and 77% respectively in case of ETR co-administration [32] potentially because of ABCB1 inhibition by ETR. This latter observation raises the question about the relative importance of CYP3A induction over ABCB1 inhibition with ETR co-administration.

The opposite effect of ETR on ATV on the one hand and on APV PK on the other might be the consequence of a greater impact of CYP3A activity on ATV PK while, considering APV, ABCB1 activity seems the most influencing factor. In our cohort, we observed that ETR decreased $[DRV]_{\text{plasma}}$ by approximately 50% by promoting a significant decrease in the $T_{1/2}$ of DRV. This PK effect is potentially further due to the ETR-mediated CYP3A-induction than the inhibition of ABCB1 activity that would theoretically have led to an increased DRV exposure. Our observation probably reflects that CYP3A activity is more importantly implicated in the PK pathway of DRV than ABCB1 and influences its $T_{1/2}$. Therefore, it appears clear that the clinical impact of this probably important drug interaction between ETR and DRV needs to be further clarified.

5. Conclusion

We have described a handy and sensitive high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method allowing the simultaneous quantification of DRV and ETR in PBMCs. This method was then applied in the largest cohort of DRV treated HIV infected patients reported to date. Our intracellular plasma ratio of DRV is lower than previously reported with the discrepancies probably due to alternative treatment regimens and/or to methodological/technical differences. ETR accumulates more efficiently in PBMCs compared to DRV.

Interestingly, we have highlighted a possible impact of ETR on DRV plasma concentrations but further investigations are needed to clarify this interaction that might incriminate the CYP3A-mediated metabolism pathway of DRV and its induction by ETR co-administration.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clinbiochem.2015.12.011>.

Conflict of interest

The authors declare that there are no conflicts of interest.

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