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# HPLC-MS/MS method for the simultaneous quantification of dolutegravir, elvitegravir, rilpivirine, darunavir, ritonavir, raltegravir and raltegravir- $\beta$ -D-glucuronide in human plasma

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## Highlight:

- This paper proposes an HPLC-MS/MS method to determine raltegravir- $\beta$ -D-glucuronide, rilpivirine, raltegravir, darunavir, dolutegravir, elvitegravir and ritonavir in human plasma using isotopic labeled internal standards.
- This method requires only 100 $\mu$ L of plasma from patients.
- This method is simple, reliable, cost-effective and suitable for routine analysis and clinical research.

**Keywords:** Liquid chromatography; Tandem mass spectrometry; Antiretroviral; Therapeutic drug monitoring

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## **Abstract**

Therapeutic drug monitoring (TDM) is essential in the optimization of antiretroviral (ARV) treatments. In this work, we describe a new method for the simultaneous quantification of six molecules: the three novel ARV agents dolutegravir (DTG), elvitegravir (ELV) and rilpivirine (RPV), the first integrase inhibitor raltegravir (RAL) and its major metabolite the raltegravir- $\beta$ -D-glucuronide (RAL-GLU), an protease inhibitor darunavir (DRV) and its booster ritonavir (RTV) in human plasma. The drugs were extracted from 100 $\mu$ L of plasma by a simple method of protein precipitation using acetonitrile. The separation was carried out on a Kinetex phenyl-hexyl column using a phase mobile composed of 55% of water (0.05% formic acid,v/v) and 45% of methanol (0.05% formic acid,v/v). The flow rate was set at 0.5mL/min. The calibration ranged from 60 to 15000 ng/mL for DRV, from 20 to 5000 ng/mL for DTG and ELV, from 10 to 2500 ng/mL for RAL, RAL-GLU, RTV and RPV. The proposed method was validated with a good precision (inter- and intra-day CV% inferior to 12.3%) and a good accuracy (inter- and intra-day bias between -9.9% and 10%) for all the analytes. The proposed method is simple, reliable and suitable for therapeutic drug monitoring (TDM) and for pharmacokinetics studies.

## 1 Introduction

Rilpivirine (RPV), dolutegravir (DTG) and elvitegravir (ELV) are the three newest licensed ARV drugs. As the second generation of non-nucleoside reverse transcriptase inhibitors (NNRTIs), RPV presents the same efficacy as efavirenz but better safety [1,2]. DTG, ELV and raltegravir (RAL) are three integrase strand transfer inhibitors (INSTI) approved by the FDA. They rapidly decrease viral loads by inhibiting the integrase enzyme which is essential for viral survival [3]. World Health Organization (WHO) currently recommends DTG-based regimen (DTG+tenofovir+emtricitabine or DTG+abacavir+lamivudine) as the preferred first-line anti-retroviral treatment for the people infected with HIV [4]. RAL is also recommended as the preferred first-line regimen for neonates, and for the infants and children in whom DTG cannot be used [4]. Finally, the combination of DTG and **darunavir (DRV)** boosted by **ritonavir (RTV)** (DRV/r) with 1 or 2 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) is recommended as an alternative second line regimen for treatment of adults and children [4].

Although, the therapeutic drug monitoring (TDM) of ARV drugs is not recommended by the guideline of U.S. department of health and human services (DHHS)[5], it has been shown in several clinical studies to be useful to optimize efficacy, avoid resistance and minimize toxicity[6–8]. The antiviral activity have been shown to associate with the trough concentration which should be  $\geq 17.65$  ng/mL for RAL[9],  $> 44.9$  ng/mL for **ELV**[10],  $> 64$  ng/mL for DTG[11] and  $> 50$ ng/mL for RPV[12].

**In France**, the RPV, DTG, ELV, DRV/r or RAL was recommended as the third agent in combination with 2 NRTI (tenofovir + emtricitabine or abacavir + lamivudine) for the naïve patients in different clinical situations[13]. These combinations of treatment were also recommended by the WHO guidelines and DHHS guidelines[4,5]. Furthermore, in previous clinical studies, the combination of DTG with RPV[14] or RAL with DRV/r [15]was recommended as treatment for the maintenance of virological suppression in experienced patients to replace the existed triple therapy, the combination of ELV with DRV was recommended for the experienced patients with multiclass resistance[16]. However, no methods were available for measuring simultaneously these ARV drugs. Perhaps that is because the challenge induced by the difference of polarity between the NRTIs and the three INSTIs or RPV. As several methods are available to measure simultaneously the tenofovir, emtricitabine, abacavir and lamivudine[17,18], an additional method for measuring RPV,

DTG, ELV, RAL, DRV and RTV is needed, especially for the three newest ARVs(RPV, DTG and ELV). Several analytical methods have been reported to determine the concentrations of these 6 ARVs using either LC-MS/MS [19–21] or HPLC-UV[22,23], and only one method available for measuring simultaneously DTG, RPV, EVL, RAL, DRV, RTV and the eleven others ARV in plasma using UPLC-MS/MS[24]. However, the main drawback of this method is the lack of isotopic labeled internal standards (IS), which could result in potential matrix effects. Furthermore, this method was not suitable for the laboratory that has not UPLC system.

Raltegravir-glucuronide (RAL-GLU) is the main metabolite of RAL[25]. A large inter- and intra-individual variability in RAL plasmatic concentrations (85.9% and 124.6% respectively) have been reported [26]. Assaying RAL-GLU plasmatic concentrations could be helpful to better understand this large inter- and intra-individual variability in RAL plasmatic concentrations. To date, clinical data including RAL-GLU plasmatic concentrations are very sparse, possibly due to the fact that only one fully validated analytical method has been reported.

The aim of this study was to develop and validate a novel analytical HPLC-MS/MS method for the simultaneous quantification of RPV, DTG, ELV, RAL, RAL-GLU, DRV and RTV in human plasma using the corresponding isotopic labeled internal standards.

## **2 Material and methods**

### **2.1 Standards and reagents**

Raltegravir potassium salt, dolutegravir, elvitegravir, [<sup>2</sup>H<sub>6</sub>]-raltegravir-glucuronide, [<sup>13</sup>C<sub>6</sub>]-rilpivirine, [<sup>13</sup>C, <sup>2</sup>H<sub>5</sub>]-dolutegravir, [<sup>13</sup>C<sub>6</sub>]-darunavir and [<sup>13</sup>C<sub>6</sub>]-elvitegravir were acquired from Alsachim (Strasbourg, France). Raltegravir-glucuronide, rilpivirine, darunavir, [<sup>2</sup>H<sub>3</sub>]-raltegravir and [<sup>2</sup>H<sub>6</sub>]-ritonavir were purchased from TRC (Toronto, Canada). Methanol (MeOH), acetonitrile (ACN), formic acid and ammonium format, all in LC/MS grade, and ritonavir were acquired from Sigma-Aldrich (St. Louis, MO, USA). LC/MS grade water was purchased from J.T.Baker (Gliwice, Poland). Drug free plasma heparinized was obtained from Utak Laboratories (Valencia, Spain) and stored at -20°C.

### **2.2 Chromatographic conditions**

Molecule separation was carried out on an Accela<sup>®</sup> System liquid chromatography (Fisher scientific, les Ulis, France) using a reversed phase Kinetex phenyl-hexyl analytical column (5  $\mu\text{m}$ , 100 mm x 3 mm; Phenomenex, Le Pecq Cedex, France) associated with a Kinetex phenyl-hexyl guard column. The column temperature was set at 30°C. Compounds were eluted using a gradient with a flow rate of 0.5 mL/min. The mobile phase consisted of water (A) and methanol (B), both with 0.05% (v/v) formic acid. The optimized gradient conditions were as follows: isocratic elution from 0 min to 1 min with 45% of B, then a linear gradient up to 100% of B from 1 min to 12 min. After holding at 100% of B during 1 min, the mobile phase composition returned to 45% of B in 0.1 min and followed by a re-equilibration step for 5 min.

Mass spectrometry detection was performed on a TSQ Quantum discovery MAX triple quadrupole mass-spectrometer (Thermo Scientific, les Ulis, France). After Heated-ElectroSpray Ionization (H-ESI), RAL-GLU and [<sup>2</sup>H<sub>6</sub>]-raltegravir-glucuronide ([<sup>2</sup>H<sub>6</sub>]-RAL-GLU) were detected in the negative multiple reaction monitoring (MRM) mode, all the other molecules were detected in the positive MRM mode. The two mass transitions with the best responses were selected for each analyte, one for quantification (first ion trace) and the second (secondary ion trace) for confirmation, and only one transition of quantification was used for each IS. Transitions, collision energies and tube lens used for each analyte and each IS were listed in the Table 1. The following parameters were optimized to achieve the best sensitivity: capillary voltage was set at 3 kV; the gas transfer temperature at 350°C; sheath gas at 45 psi, and auxiliary gas at 35 psi. Nitrogen employed as both nebulizing and auxiliary gas was supplied by a NM 30 LA generator (LabGaz System, Massy, France).

Xcalibur software (version 2.0, Thermofisher Scientific) was used for instrument control, data acquisition and analysis.

### **2.3 Preparation of calibration standards and quality control samples**

Stock solutions of RPV, DTG, ELV, RAL, RAL-GLU, DRV and RTV were separately prepared for calibration standards (CALs) and quality control samples (QCs) in LC/MS grade methanol at a final concentration of 1mg/mL. Stock solutions of [<sup>13</sup>C<sub>6</sub>]-darunavir ([<sup>13</sup>C<sub>6</sub>]-DRV), [<sup>13</sup>C,<sup>2</sup>H<sub>5</sub>]-dolutegravir ([<sup>13</sup>C,<sup>2</sup>H<sub>5</sub>]-DTG), [<sup>13</sup>C<sub>6</sub>]-elvitegravir ([<sup>13</sup>C<sub>6</sub>]-ELV), [<sup>2</sup>H<sub>6</sub>]-RAL-GLU, [<sup>2</sup>H<sub>3</sub>]-raltegravir ([<sup>2</sup>H<sub>3</sub>]-RAL), [<sup>2</sup>H<sub>6</sub>]-ritonavir ([<sup>2</sup>H<sub>6</sub>]-RTV) and [<sup>13</sup>C<sub>6</sub>]-rilpivirine

( $[^{13}\text{C}_6]$ -RPV) were also prepared in LC/MS grade methanol at a concentration of 1mg/mL. All these stock solutions were aliquoted, then stored at  $-20^\circ\text{C}$  in darkness.

The day of analysis, two working solutions which contain 6 and 150  $\mu\text{g/mL}$  of **DRV**, 2 and 50  $\mu\text{g/mL}$  of DTG and ELV, 1 and 25  $\mu\text{g/mL}$  of RAL, RAL-GLU, RTV and RPV were diluted with appropriate volume drug-free plasma to prepare CALs. A set of eight CALs with **DRV** in the range of concentrations from 60 to 15000 ng/mL, with DTG and ELV in the range from 20 to 5000 ng/mL, with RAL, RAL-GLU, RTV and RPV in the range from 10 to 2500 ng/mL was prepared.

Four levels of QCs for lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC) and high quality control (HQC) were prepared freshly at concentrations of 60, 180, 6000, 12000 ng/mL for **DRV**; 20, 60, 2000 and 4000 ng/mL for DTG and ELV; 10, 30, 1000 and 2000 ng/mL for RAL, RAL-GLU, RTV and RPV respectively.

An IS working solution of 625 ng/mL for  $[^2\text{H}_6]$ -RAL-GLU,  $[^2\text{H}_3]$ -RAL,  $[^2\text{H}_6]$ -RTV and  $[^{13}\text{C}_6]$ -RPV, and 1250 ng/mL for  $[^{13}\text{C}_6]$ -DRV,  $[^{13}\text{C}, ^2\text{H}_5]$ -DTG and  $[^{13}\text{C}_6]$ -ELV was freshly prepared by dilution from stock solution of each IS in LC/MS grade water.

## **2.4 Sample preparation**

A volume of 100  $\mu\text{L}$  of plasma of each sample was mixed with 20  $\mu\text{l}$  of IS working solution. Then, 300  $\mu\text{l}$  of acetonitrile was added to precipitate the proteins. The mixture was vortexed for 5 minutes (VX-2500 Multi-Tube Vortexer<sup>®</sup>, VWR, **Fontenay-sous-Bois, France**), then centrifuged at 17000 g at  $4^\circ\text{C}$  for 10 minutes. The supernatants were transferred into glass tubes and evaporated to dryness at room temperature under a gentle nitrogen stream. Two hundred microliters of mobile phase were added to reconstitute the dry residue. After 10 minutes of centrifugation at 17000 g at  $4^\circ\text{C}$ , 25  $\mu\text{l}$  of supernatant of each sample was injected and analyzed in the LC-MS/MS system.

## **2.5 Method validation**

The method was validated according to the Food and **Drug** Administration (FDA) [27] and European Medicines Agency (EMA) [28] guidelines for bioanalytical method validation.

### **2.5.1 LLOQ, specificity, selectivity and cross-talk**

LLOQ of each molecule was determined based on the signal-to-noise ratio which was at least of 10:1. Selectivity of the method was confirmed by analyzing 10 drug-free human plasmas to rule out the risk of interference with endogenous compounds. Specificity of the method was also investigated by analyzing 39 potential co-medications including 19 antibiotics (amoxicillin, cefazolin, cefepime, cefotaxime, ceftazidime, cloxacillin, ciprofloxacin, flucytosine, levofloxacin, isoniazid, meropenem, moxifloxacin, rifampicin, sulfamethoxazole, trimethoprim, piperacillin, paracetamol, pefloxacin and pyrazinamide), 4 antifungics (fluconazole, itraconazole, voriconazole and posaconazole), 12 ARVs (lamivudine, tenofovir, maraviroc, abacavir, efavirenz, lopinavir, cidofovir, emtricitabine, zidovudine, ganciclovir, nevirapine and atazanavir) and 4 antiepileptics (clobazam, lamotrigine, levetiracetam and carbamazepine) at plasma therapeutic levels. The MRM signal of interference for each analyte should be less than 20% of the LLOQ for the corresponding analytes and less than 5% for the corresponding IS.

The cross-talk study was investigated between the analytes and the respective isotope IS. The contribution of the IS to the response of each analyte was evaluated by analyzing extracted blank plasma spiked with only IS in five replicates. The MRM signal in the transition window of each analyte should be  $\leq 20\%$  of the LLOQ. The contribution of the analytes to the response of each IS was evaluated by analyzing the highest standards samples without IS in five replicates. The MRM signals in the transition window of each IS should be  $\leq 5\%$  of the IS in the LLOQ.

### **2.5.2 Linearity, accuracy and precision**

The linearity of the method was evaluated by analyzing three complete standard curves on three different days. With each standard curve, four levels of QCs were assayed with five replicates. Calibration curves, which were plotted using the ratio of peak area of analyte/peak area of IS versus analyte concentration, were calculated using either linear regression with weight of  $1/X^2$  for RAL-GLU, RAL, DTG, ELV and RTV or quadratic regression with weight of  $1/X$  for RPV and DRV. Repeatability and reproducibility of the method were evaluated by analyzing the intra- and inter-day precision (coefficient of variation, expressed as CV%) and accuracy (expressed as bias) of four levels of QCs. Five replicates of each level of QCs were assayed in one run for the intra-day study and in three runs on three different days for the

inter-day study. The CV and bias should be within  $\pm 15\%$  except LLOQ which should not exceed 20%. The carry-over study was performed with three replicates by injecting two extracted drug-free plasma after the injection of an upper limit of CALs.

### **2.5.3 Recovery, matrix effect, and carry over**

Absolute recovery of the analytes and IS was determined by comparing the mean peak areas of extracted plasma samples to those of reference standards. The reference standards were prepared by directly diluting the working solution of all the analytes in the extracted blank plasma at the same concentration. Absolute matrix effect of the method was investigated in 6 different plasmas using the post-extraction addition method described by Matusezowski et al[29]. The matrix effect was obtained by comparing the mean peak areas of the analytes directly diluted in extracted blank plasma with the peak areas of the analytes diluted in phase mobile at the same concentration. For recovery and matrix effect tests, two concentrations at LQC (30ng/mL for RAL-GLU, RPV, RAL and RTV, 60 ng/mL for DTG and ELV, and 180ng/mL for **DRV**) and HQC (2000 ng/mL for RAL-GLU, RPV, RAL and RTV, 4000 ng/mL for DTG and ELV, and 12000 ng/mL for **DRV**) were analyzed with six replicates for each lot of plasma in a single run. According to the EMA guideline, a matrix effect  $\leq 15\%$  was considered to be negligible, and the CV should not be greater than 15%. Furthermore, the matrix effect was also evaluated by comparison of slopes of calibration curves prepared in four different lots of plasma for each analytes.

The carry over study was performed by injecting two extracted blank plasmas after the injection of the upper limit of CAL. The interference should not exceed 20% of the LLOQ and 5% of the IS.

### **2.5.4 Stability**

Different stability tests for these analytes have been already conducted in previous studies. Thus, all the analytes are known to be stable after at least 3 freeze-thaw cycles. Furthermore, plasma can be stored at room temperature for 24 hours without any significant change in drug level [19,25,30]. For long term stability study, all the other analytes, except RAL-GLU (no data available), have already been reported to be stable at  $-20^{\circ}\text{C}$  for at least 6 weeks [23,31,32]. Short-term stability of the whole blood stability ( $+4^{\circ}\text{C}$ ) and processed samples

(+4°C and room temperature) for 24 hours, and long-term stability (-20°C for 1 month) of the unprocessed plasma samples were investigated in our study. Two levels of concentration at LQC and HQC for all the analytes were analyzed in triplicate, for each stability test. The stability of stock solution of each analyte was investigated at -20°C for 6 months. The concentrations observed at the end of the stability study condition were compared with those of the beginning. The bias and CV should not exceed 15% for this stability test.

## **2.6 Application**

This method was confirmed by analyzing the external quality control samples from ASQUALAB (Paris, France). This program of external quality control was confirmed by ISO 9001. More than twenty laboratories took part in the program for analyzing the different ARV. Our results were compared with the mean concentrations obtained by all the participants. With the Z-score less than 2 and the bias less than 25% for the two different level of external quality control, the method could be considered to be reliable [33].

The proposed method has been successfully applied to the plasmas of routine TDM from HIV-infected patients and to the RalFE ANRS 160 study. The RalFE ANRS 160 study was an open-label, multicenter, phase II randomized trial which was approved by the French Ethics Committee. In this study, all the women received 400 mg raltegravir twice daily with optimized background therapy. A signed consent was obtained from each pregnant woman. The aim was to study pharmacokinetic properties of raltegravir in pregnant women infected by HIV-1, during the third trimester of pregnancy (between 30 and 37 weeks of amenorrhea) and 1 month after childbirth.

## **3 Results and discussion**

### **3.1 Method development**

As mentioned in several previous studies[25,31,34,35], a part of RAL-GLU was degraded to RAL in source using LC-MS/MS, this transformation phenomenon were likely not avoidable since no solution have been reported. However, with the same transformation rate in the calibration standards and plasma of patients, the concentration of RAL-GLU would not be impacted by this transformation phenomenon. To avoid the interference of RAL-GLU in RAL quantification, baseline chromatographic separation was obligatory required. Several different

types of reversed phase columns have been tested. Finally, the kinetex phenyl-hexyl column (100\*3mm, 5 $\mu$ m) was chosen in order to have sharp and symmetric peaks, correct retention times, and most importantly, a good separation for all the analytes. For the mobile phase, as ACN had an interaction effect with the phenyl-hexyl column, methanol was chosen in this method. As previously reported for LC-MS/MS method [24,25,36], 0.05% formic acid were added in the mobile phase (water and methanol) to optimize the ionization of these molecules. In these chromatographic conditions, the retention times were 2.8 minutes for RAL-GLU, 4.0 minutes for RPV, 5.3 minutes for RAL, 6.1 minutes for DRV, 6.7 minutes for DTG, 9.1 minutes for ELV and 9.4 minutes for RTV. The LLOQs of all the analytes in our method are at least 2 fold lower than the concentration minimum requested for therapeutic monitoring according to the different guidelines (LLOQ/recommendation: 10/50 ng/mL for RPV, 10/30 ng/mL for RAL, 20/64 ng/mL for DTG, 60/500 ng/mL for DRV et 20/45 for ELV) [10–13]. Furthermore, the LLOQs used in our method are similar compared with the LLOQs used in the previous studies [19–21,24]. The chromatogram from blank plasma samples and LLOQ of each analytes were shown in FIG 1. The weight of  $1/X^2$  was used for calibrating the calibration curve of RAL-GLU, RAL, DTG, ELV and RTV. Compared to the  $1/X$ , with weight of  $1/X^2$ , we have better results of intra- and inter-day accuracy, especially for the low concentrations. Probably because our LLOQ is relatively low. In another study, the weight of  $1/X^2$  was recommended for calculating the calibration range with low concentration[37].

## **3.2 Method Validation**

### **3.2.1 LLOQ, specificity,selectivity and cross-talk**

The LLOQ was 10 ng/mL RAL-GLU, RPV, RAL and RTV, 20 ng/mL for DTG and ELV, and 60 ng/mL for DRV.

For the specificity analysis, 39 drugs (listed in the section 2.5) were tested. Selectivity of the method was also investigated by analyzing 10 different blank plasmas. No significant interference from exogenous or endogenous compounds was found in both tests of specificity and selectivity.

No cross-talk between the analytes and IS was observed.

### **3.2.2 Linearity, accuracy and precision**

Calibration curves were linear and ranged from 10 to 2500 ng/mL for RAL-GLU, RAL, RTV and RPV, from 20 to 5000 ng/mL for DTG and ELV, and from 60 to 15000 ng/mL for DRV. The correlation coefficients ( $r^2$ ) of the calibration curves exceeded 0.995 for all the analytes showing a good linearity of the method for each analyte. The values of accuracy and precision were below 10% and 13% for the intra- and inter-day studies, respectively (Table 2). Taken together, these results indicate the reliability of the present method.

### **3.2.3 Recovery, matrix effects and carry over**

The recovery of each analyte obtained by analyzing LOQ and HOQ were 96% and 91% for RAL-GLU, 103% and 100% for RPV, 102% and 94% for RAL, 101% and 100% for DRV, 101% and 98% for DTG, 105% and 99% for ELV, 111% and 101% for RTV, respectively. These results of recovery was similar with the previously reports [19,24,25,38] using ACN or ANC plus methanol precipitation methods.

The absolute matrix effects (n=6 different plasmas) were between 87-111% for RAL-GLU, 103-111% for RLV, 94-106% for RAL, 98-108% for DRV, 96-106% for DTG, 97-108% for ELV and 102-115% for RTV, which suggests that no ionization enhancement or suppression was observed. The matrix effect was also confirmed by the similar slopes of calibration curves prepared in different lots of plasma shown in table 3.

No carry over effect was observed for all analytes and IS.

### **3.2.4 Stability**

All the analytes and IS were considered to be stable in stock solution at -20°C for at least 6 months. The bias value were between -8.9% to 8.7%. The results of the other stability studies were presented in Table 4. With bias between -13% to 15%, all the analytes could be considered to be stable in mobile phase after being processed at autosampler temperature (8°C) and at room temperature during 24 hours and in plasma at -20°C for 1 month. For the stability in whole blood, all the analytes were stable at room temperature during 24 hours, except dolutegravir which was stable at least 3 hours. At least 50% of degradation was observed for dolutegravir in whole blood after 24 hours and 24% after 7 hours at room temperature.

### 3.3 Application

Since 2019, one low concentration level and one high concentration level of external quality control samples were analyzed for DRV and RTV. The Z-scores (bias) obtained were -0.21 (-1.7%) and -1.3 (-0.12%) for the low concentration level of DRV and RTV, 1.9 (18.3%) and 0.42 (5.3%) for the high concentration level of DRV and RTV, respectively. These results confirm the reliability of the proposed method.

In the RalFE ANRS 160 study, a total of 39 trough concentrations ( $C_{\text{trough}}$ ) of raltegravir and raltegravir-glucuronide from 39 pregnant women during the third trimester were obtained. The median  $C_{\text{trough}}$  raltegravir and raltegravir-glucuronide were 77 ng/mL and 112 ng/mL respectively, with a range from 11 to 749 ng/mL for raltegravir and from 23 ng/mL to 1310 ng/mL for raltegravir-glucuronide. This found is in accordance with the only two previous studies of pharmacokinetics of raltegravir in pregnancy [39,40], in which the median  $C_{\text{trough}}$  (range) of raltegravir in the third trimester of pregnancy was found to be 77 ng/mL (43 to 137 ng/mL) by Blonk et al.[39] in PANNA study and to be 64 ng/mL (11 to 607 ng/mL) by Watts et al. [40] in IMPAACT 1026s study.

In this study, 6 women received 600 mg darunavir and 100mg ritonavir twice daily as the co-treatment with raltegravir during the third trimester of pregnancy. The trough concentrations of RAL, RAL-GLU, DRV and RTV of these 6 pregnant women were shown in table 5. The median  $C_{\text{trough}}$  (interquartile range) of darunavir and ritonavir obtained were 2319 ng/mL (1499-3485 ng/mL) and 293 ng/mL (183-319 ng/mL). This result is comparable with the result obtained from the HIV-infected women during the third trimester of pregnancy by Stek et al. in IMPACCT network Protocol P1026 study [41] in which the median  $C_{\text{trough}}$  (interquartile range) of darunavir and ritonavir were found to be 2220 ng/mL (1680-3260ng/mL) and 180 ng/mL (110-250 ng/mL) respectively. A chromatogram of one of the six pregnant women receiving a combination of raltegravir/darunavir/ritonavir at the dosage of 400mg/600/mg/100mg twice daily was show in Fig. 2. The concentrations obtained were 449 ng/mL, 77 ng/mL, 1499 ng/mL and 289 ng/mL for raltegravir, raltegravir-glucuronide, darunavir and ritonavir respectively.

## 4 Conclusion

Here, we present the first fully developed and validated HPCL-MS/MS method allowing the simultaneous quantification of RAL, RAL-GLU, DGT, ELV, RLV, DRV and RTV in human plasma samples. To the best of our knowledge, only one fully validated method [24] has been published, which simultaneously quantifies RAL, DGT, ELV, RLV, DRV and RTV on UPLC-MS/MS using quinoxalone as IS for all the analytes. But no HPLC method was available. The proposed method could contribute to the TDM or pharmacokinetics studies of these molecules in laboratories having no UPLC-MS/MS. Furthermore, our study uses isotopic labeled internal standards, a “gold reference” when quantification is performed using a mass spectrometry detector. Although, it is more expensive, it could better respect the analytes and avoid potential matrix effect from co-eluting endogenous or exogenous compounds which could be present in blood samples from some patients[38]. Furthermore, the proposed method needs only 100 $\mu$ L of patients’ plasma samples which simplify the collection of blood samples from the children.

This method is proposed to quantify three INSTIs (RAL, DTG and ELV) which are not co-administrated in HIV patients. However, these three INSTIs could be used in combination with DRV/r in different cases. Furthermore, in the guidelines of WHO, DHHS and France[4,5,13], RAL, DTG, ELV, DRV/r, and RPV have the same co-treatments (tenofovir+emtricitabine /lamivudine or abacavir+emtricitabine). The proposed method could be used as an additional method to the existing method which quantified tenofovir, emtricitabine, abacavir and lamivudine in the laboratory for routine use. Another limitation of this method is the long analytical run time (18min). Even if the run time is long, it was considered to be acceptable for routine practices to quantify 7 analyte using a HPLC system but not an UPLC system.

The proposed method is precise, accurate, specific and sensitive, suitable for the TDM of routine clinical practice and pharmacokinetic studies of raltegravir-glucuronide, raltegravir, dolutegravir, elvitegravir, rilpivirine, darunavir and ritonavir.

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### Figure description

Fig 1. Typical chromatogram of blank extracts and LLOQ of each analyte : raltegravir- $\beta$ -D-glucuronide (Ral-Glu, 10 ng/mL) at 2.8 min, rilpivirine (10 ng/mL) at 4.0 min, raltegravir (10 ng/mL) at 5.3 min, darunavir (60 ng/mL) at 6.1 min, dolutegravir (20 ng/mL) at 6.7 min, elvitegravir (20 ng/mL) at 9.1 min and ritonavir (RTV, 10 ng/mL) at 9.4 min with absolute abundance (cps) in Y axis and retention time (min) in X axis.

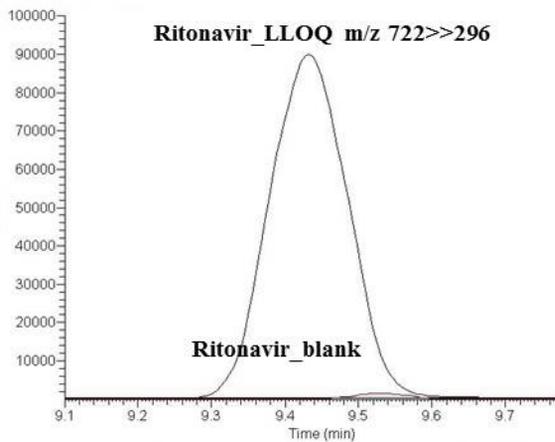
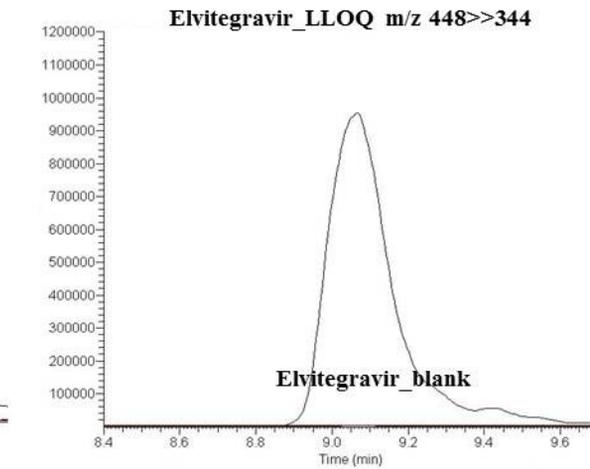
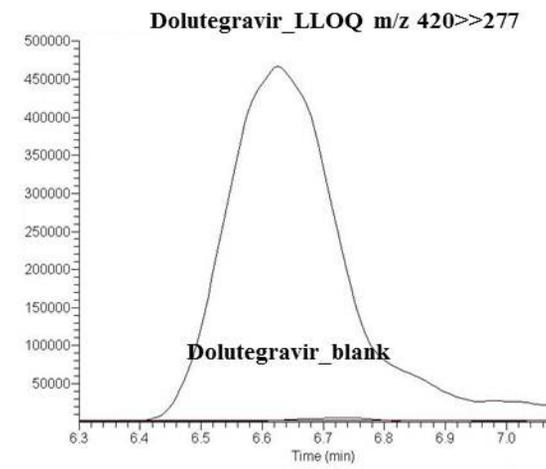
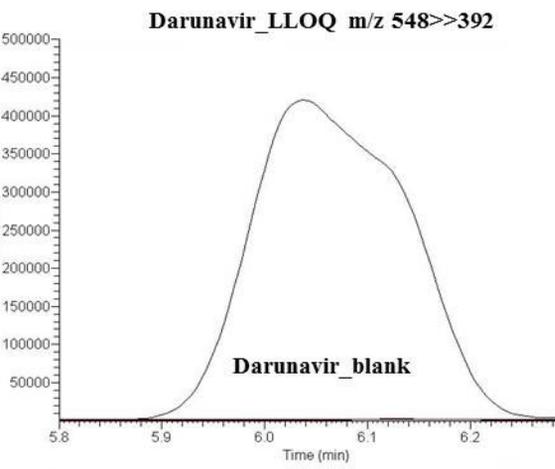
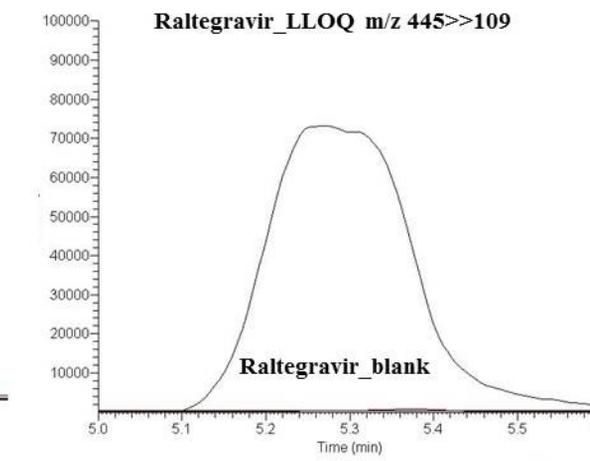
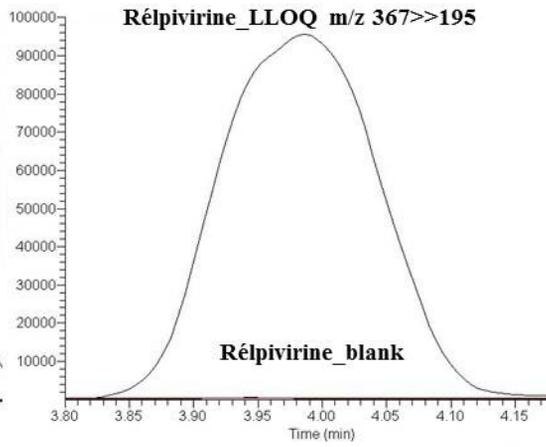
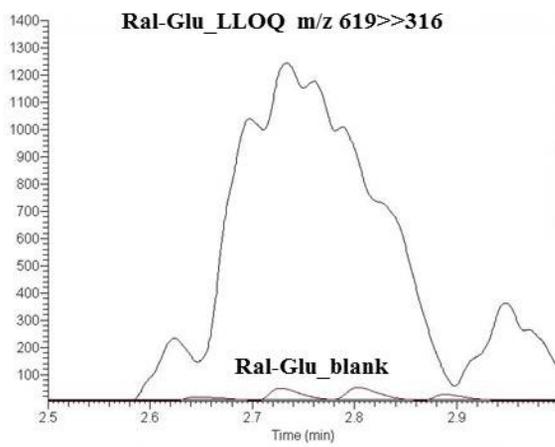
Fig 2. Chromatogram of a  $C_{\text{trough}}$  plasma sample from a patient receiving a combination treatment of raltegravir 400 mg twice-daily (concentration of raltegravir: 449ng/mL and concentration of raltegravir-glucuronide: 77ng/mL), darunavir 600 mg twice-daily (concentration: 1499 ng/mL) and ritonavir 100 mg twice daily (concentration: 289 ng/mL), with relative abundance in Y axis and retention time in X axis.

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### Competing Interests

There are no competing interests to declare.



Ral-Glu

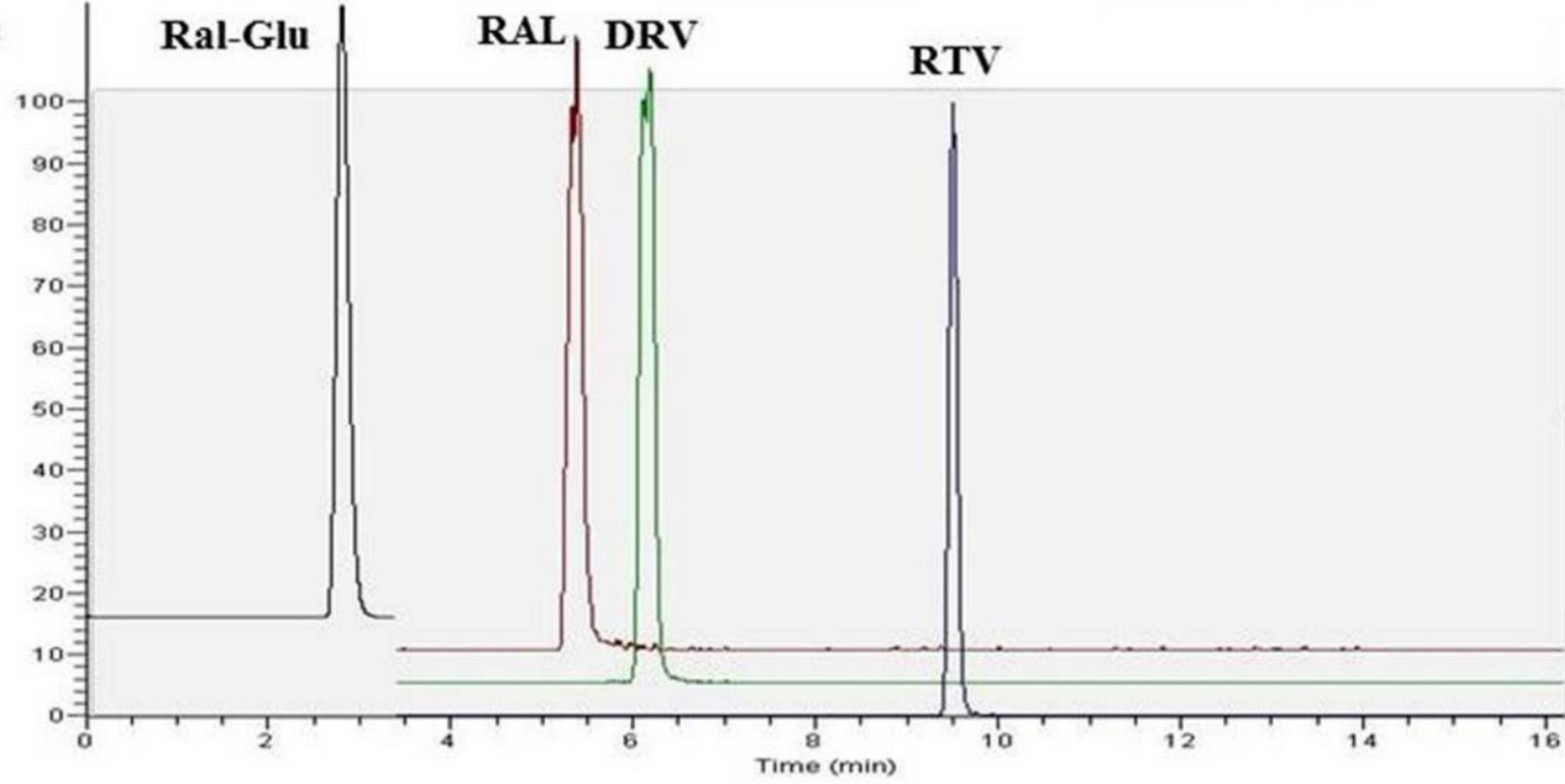
RAL

DRV

RTV

100  
90  
80  
70  
60  
50  
40  
30  
20  
10  
0

Time (min)



**Table.1** Parent to fragment (daughters) mass transition and relative collision energies (CE), tube lens used for each analyte and internal standard using HPLC-MS/MS

Analytes	Transition (m/z)	Collision energy (V)	Tube Lens (V)	Retention time (min)	
Raltegravir-glucuronide	Quantification	619.100>>316.160	32	60	2.8
	Confirmation	619.100>>443.210	19		
Rilpivirine	Quantification	367.102>>195.090	33	97	4.0
	Confirmation	367.102>>128.050	60		
Raltegravir	Quantification	445.117>>108.970	33	76	5.3
	Confirmation	445.117>>361.110	16		
Darunavir	Quantification	548.156>>392.190	13	58	6.1
	Confirmation	548.156>>436.180	5		
Dolutegravir	Quantification	420.100>>277.070	33	92	6.7
	Confirmation	420.100>>126.980	60		
Elvitegravir	Quantification	448.072>>344.050	29	84	9.1
	Confirmation	448.072>>430.150	20		
Ritonavir	Quantification	721.600>>296.090	17	84	9.4
	Confirmation	721.600>>268.060	28		
[ <sup>2</sup> H <sub>6</sub> ]-Raltegravir-glucuronide	Quantification	625.070>>322.200	31	55	2.8
[ <sup>13</sup> C <sub>6</sub> ]-rilpivirine	Quantification	373.140>>195.140	35	90	4.0
[ <sup>2</sup> H <sub>3</sub> ]-raltegravir	Quantification	448.136>>108.960	34	73	5.3
[ <sup>13</sup> C <sub>6</sub> ]-darunavir	Quantification	554.177>>398.230	13	62	6.1
[ <sup>13</sup> C, <sup>2</sup> H <sub>5</sub> ]-dolutegravir	Quantification	426.144>>277.060	28	113	6.7
[ <sup>13</sup> C <sub>6</sub> ]-elvitegravir	Quantification	454.124>>350.040	32	76	9.1
[ <sup>2</sup> H <sub>6</sub> ]-ritonavir	Quantification	725.120>>300.120	33	84	9.5

**Table 2.** Precision and accuracy of raltegravir-glucuronide, rilpivirine, raltegravir, darunavir, dolutegravir, elvitegravir and ritonavir in human plasma

	Theoretical ( $\mu\text{g/L}$ )	Intra-day (n=5)		Inter-day (n=15)	
		Bias* (%)	Precision (%)	Bias* (%)	Precision (%)
<b>Raltegravir- glucuronide</b>	10	5.2	8.4	9.1	7.7
	30	-9.9	5.9	-0.9	9.3
	1000	-1.3	5.1	1.4	4.0
	2000	6.1	7.8	5.9	5.6
<b>Rilpivirine</b>	10	-5.0	9.0	-2.5	12.3
	30	-9.5	6.9	-1.4	9.3
	1000	-3.4	5.1	0.5	6.2
	2000	7.0	6.8	4.2	5.8
<b>Raltegravir</b>	10	4.3	8.1	5.9	7.2
	30	-7.6	6.4	-7.6	7.4
	1000	4.1	6.0	3.6	4.0
	2000	10.0	6.7	7.1	5.9
<b>Darunavir</b>	60	4.9	5.4	6.0	12.1
	180	-8.4	3.3	-4.4	7.8
	6000	-2.8	4.9	-4.3	5.0
	12000	7.8	7.6	0.9	8.3
<b>Dolutegravir</b>	20	-7.3	5.1	1.0	8.0
	60	-7.2	6.0	-1.8	8.9
	2000	0.2	3.2	2.5	4.9
	4000	1.9	9.2	3.8	7.5
<b>Elvitegravir</b>	20	-6.8	5.8	2.0	10.4
	60	-8.6	3.5	-2.9	7.9
	2000	1.8	4.3	1.9	4.7
	4000	9.2	7.3	6.6	6.1
<b>Ritonavir</b>	10	-1.9	8.2	2.1	11.1
	30	-9.5	5.7	-6.6	7.5
	1000	-1.1	4.5	-2.5	4.3
	2000	8.7	7.5	2.1	8.5

\*Accuracy is expressed as bias

Table 3 Slopes of calibration curves prepared in different lots of plasma for raltegravir-glucuronide, rilpivirine, raltegravir, darunavir, dolutegravir, elvitegravir and ritonavir.

	Plasma lot 1	Plasma lot 2	Plasma lot 3	Plasma lot 4	CV
Raltegravir-glucuronide	0.0016	0.0016	0.0016	0.0015	3.2%
Rilpivirine	0.0035	0.0033	0.0034	0.0035	2.8%
Raltegravir	0.00068	0.00064	0.00067	0.00067	2.1%
Darunavir	0.00085	0.00077	0.00081	0.00087	5.4%
Dolutegravir	0.0012	0.0011	0.0012	0.0012	4.1%
Elvitegravir	0.0011	0.0012	0.0011	0.0012	5.0%
Ritonavir	0.0083	0.0088	0.0092	0.0081	5.8%

**Table 4** Stability of raltegravir-glucuronide, rilpivirine, raltegravir, darunavir, dolutegravir, elvitegravir and ritonavir under various storage conditions (n=4 for each value)

	processed samples					unprocessed samples		Whole blood samples	
	Concentration (mg/L)	At room temperature for 24 hours		In autosampler (at 8°C) for 24 hours		At -20°C for 1 month		At room temperature for 24 hours	
		Stability <sup>1</sup> (%)	CV <sup>2</sup> (%)	Stability <sup>1</sup> (%)	CV <sup>2</sup> (%)	Stability <sup>1</sup> (%)	CV <sup>2</sup> (%)	Stability <sup>1</sup> (%)	CV <sup>2</sup> (%)
<b>Raltegravir-glucuronide</b>	30	98.3	4.3	-12.8	13.4	96.2	1.4	95.1	1.8
	2000	102.8	4.5	7.2	3.5	103.2	1.5	108.0	10.2
<b>Rilpivirine</b>	30	97.7	4.6	3.6	14.2	97.1	3.2	91.6	5.7
	2000	97.3	5.1	-2.1	4.4	105.5	7.7	100.8	6.2
<b>Raltegravir</b>	30	92.6	3.9	-12.9	2.2	104	2.1	91.6	5.2
	2000	106.1	1.1	9.6	3.5	106.5	0.5	97.0	6.2
<b>Darunavir</b>	180	104.6	5.0	-1.5	4.0	99.2	1.5	89.4	7.1
	12000	101.7	5.1	3.2	3.0	104.8	1.3	95.1	6.4
<b>Dolutegravir</b>	60	92.6	3.4	-9.3	0.3	96.2	1.8	88.3 <sup>3</sup>	11.9 <sup>3</sup>
	4000	97.4	5.4	5.3	1.5	99.3	1.1	95.2 <sup>3</sup>	2.5 <sup>3</sup>
<b>Elvitegravir</b>	60	94.8	0.5	-6.1	0.6	103.7	6.9	95.6	5.9
	4000	101.7	2.4	1.2	1.3	114	7.6	97.2	7.2
<b>Ritonavir</b>	30	107.1	5.3	14.8	9.7	105.3	1.1	92.6	7.4
	2000	105.6	4.1	5.9	6.7	115	6.8	104.7	9.7

<sup>1</sup> Expressed as the mean percentage change from time zero (nominal concentration)

<sup>2</sup>CV: coefficient variation

<sup>3</sup>Stability for 3 hours

**Table 5** The  $C_{\text{trough}}$  of **raltegravir, raltegravir-glucuronide, darunavir and ritonavir** in six pregnant women

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
<b>Raltegravir</b> (ng/mL)	77	24	187	14	69	45
<b>Raltegravir-glucuronide</b> (ng/mL)	449	30	1310	19	97	176
<b>Darunavir</b> (ng/mL)	1499	925	4099	2534	3485	2104
<b>Ritonavir</b> (ng/mL)	230	183	824	108	297	319

100µL Plasma + 300µL Acetonitril



Centrifugation  
Evaporation



Reconstitution



Centrifugation  
Injection



Analyse

