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Short communication

Quantification of raltegravir (MK0518) in human plasma by high-performance liquid chromatography with photodiode array detection

Lauriane Goldwirt, Aurélie Barrail-Tran, Maria Da Cruz, Anne-Marie Taburet, Valérie Furlan*

Pharmacie Clinique, Laboratoire de Toxicologie, CHU de Bicêtre AP-HP, 78 avenue du Général Leclerc, 94270 Le Kremlin-Bicêtre, France

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ABSTRACT

A precise and accurate high-performance liquid chromatography (HPLC) method with photodiode array detection has been developed and validated for raltegravir, a human immunodeficiency virus integrase strand transfer inhibitor (HIV-1 INSTI). Plasma (300 μL) was extracted with dichloromethane/hexane 50:50 (v/v) after addition of the internal standard, 6,7-dimethyl-2,3-di(2-pyridyl) quinoxaline. The compounds were separated using a dC18 column and detected with ultraviolet detection at 320 nm. The limit of quantification was 10 ng/mL for raltegravir. The method was linear and validated over a concentration range of 0–10,000 ng/mL. The intra-day precision ranged from 3.1 to 12.3%, while the intra-day accuracy ranged from −15.0 to −0.5%, the inter-day precision and accuracy were less than 7%. The mean recovery was 76.8%. Application to clinical samples taken from patients treated with raltegravir indicated that the method is suitable for measuring plasma concentrations of raltegravir in pharmacokinetic studies of clinical trials.

1. Introduction

Raltegravir (MK0518, Isentress®) is a human immunodeficiency virus integrase strand transfer inhibitor (HIV-1 INSTI) indicated in treatment-experienced adult patients who have evidence of viral replication and HIV-1 strains resistant to multiple antiretroviral agents (400 mg bid). The efficacy of raltegravir after administration to HIV-infected patients whose virus is resistant to at least 3 classes of antiretroviral drugs has been clinically proven in recent Benchmark 1 and 2 clinical trials [1,2]. The number of patients who responded, the rapidity and durability of the antiretroviral response on exposure to raltegravir make this drug extremely powerful. No therapeutic range has yet been established for raltegravir, but the IC95 is 33 nM [3] (close to 15 ng/mL). Raltegravir is metabolized by glucuronidation involving UGT1A1 isoenzyme [4]. The pharmacological and clinical impact of a modification of exposure to raltegravir coadministered with other drugs (antiretroviral combination, prophylactic antimicrobial therapy) has to be assessed in HIV-infected patients, as the UGT1A1 activity may be increased by enzymatic inducers such as rifampicin [5], or decreased by specific enzymatic inhibitors such as atazanavir [6]. Consequently, a bioanalytical method to determine the concentration of raltegravir in human plasma was required to support clinical development studies. To date nine HPLC analytical methods for quantification of raltegravir have been published. Five of these use liquid chromatography–mass spectrometry [4,7–10], one uses fluorescence detection [11], and three others use ultraviolet (UV) detection after a liquid–solid phase [12–14]. Here we describe the validation of a simple, sensitive and cost effective HPLC method using photodiode array (PDA) detection and liquid–liquid extraction for quantification of raltegravir.

2. Experimental

2.1. Reagents

Acetic acid 99–100% was from Fisher Scientific (EC 200-580-7; Elancourt, France). Acetonitrile Chromasolv® for HPLC gradient grade (EC 200-835-2) and sodium acetate Sigma ultra min 99% (EC 204-823-8) were from Sigma–Aldrich (Steinheim, Germany). Raltegravir potassium salt was kindly supplied by Merck, and 6,7-dimethyl-2,3-di(2-pyridyl) quinoxaline was purchased from Sigma–Aldrich (EC 229-592-0, Steinheim, Germany).

2.2. Instrumentation

The HPLC system consisted of a LC 10 AD VP Shimadzu pump, a Waters 996 photodiode array detector, and a Waters 717 plus auto sampler used at ambient temperature. Empower 2.0 (Waters software) was used for data acquisition and processing.

* Corresponding author at: Bicêtre Hospital, Clinical Pharmacy, 78 rue du General Leclerc, 94270 Le Kremlin-Bicêtre, France. Tel.: +33 01 45 21 29 64; fax: +33 01 45 21 28 60.
E-mail addresses: aurelie.barrail-tran@bct.aphp.fr (A. Barrail-Tran), anne-marie.taburet@bct.aphp.fr (A.-M. Taburet), valerie.furlan@bct.aphp.fr (V. Furlan).

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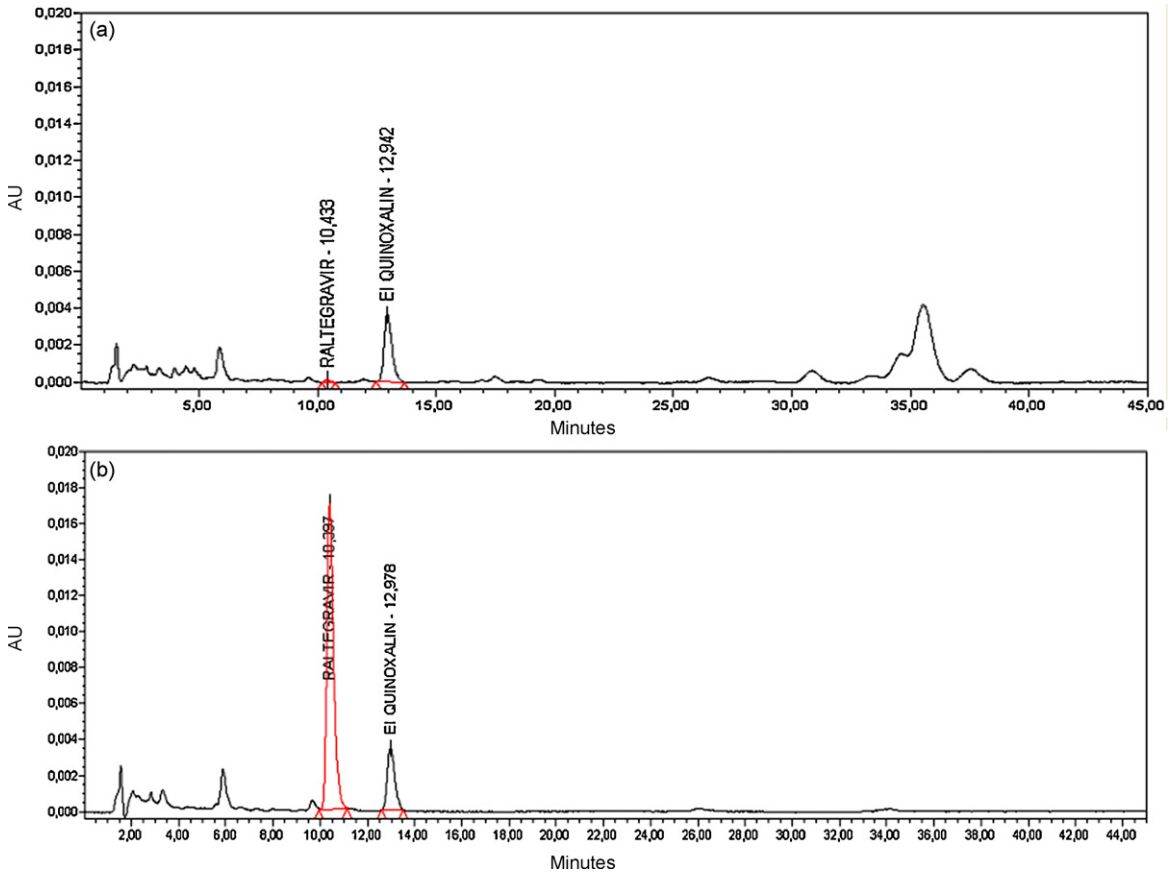

Fig. 1. Representatives chromatograms of the 10 ng/mL LOQ (a) and a patient treated by raltegravir (1254 ng/mL) (b).

2.3. Chromatographic conditions

Chromatography was performed on a 2.1 mm × 150 mm Atlantis® dC18 (3 μm) column with mobile phase consisting of 710/290 (v/v, %) acetate buffer (0.1 M, pH 4)/acetonitrile with a 0.4 mL/min flow rate. The PDA detector was set at 320 nm. A 80 μL full loop injection was used with a total run time of 45 min.

2.4. Preparation of calibration standards and quality control samples

2.4.1. Stock and diluted standard solutions

Two different 500 μg/mL stock solutions of raltegravir were prepared in water. After aqueous dilution of these raltegravir stock solutions, working standard solutions (50 and 5 μg/mL) aliquoted in polypropylene tubes and stored at −20 °C, were used to prepare calibration standards and quality controls. Internal standard solution of quinoxaline was prepared at 0.5 μg/mL in methanol.

2.4.2. Preparation of calibration standards and quality controls

Calibration standards of raltegravir ranged from 10 to 10,000 ng/mL and were prepared by adding an appropriate volume of stock or aqueous diluted solution (50 and 5 μg/mL) to aliquots of blank human plasma. Low, middle and high quality controls at 75, 1500 and 7500 ng/mL were prepared by diluting a separate 500 μg/mL raltegravir solution or aqueous diluted solutions (50 and 5 μg/mL) in aliquots of blank human plasma. Aliquots of calibration standard and quality control were transferred into polypropylene tubes and stored at −20 °C.

2.5. Extraction procedure

Plasma samples (300 μL) were pipetted into a glass tube and spiked with 100 μL of a 0.5 μg/mL working internal standard solution. Two milliliters of a dichloromethane/hexane (50/50, v:v) mixture and 200 μL of acetate buffer (0.1 M, pH 4) were added. After vortex-mixing for 4 min, tubes were centrifuged at 4000 rpm for 10 min. The organic layer was transferred into a glass tube and evaporated to dryness under a nitrogen stream at ambient temperature for 15 min. The dry residue was reconstituted in 100 μL of the mobile phase, mixed, and 80 μL were injected into the chromatographic system for analysis.

2.6. Data evaluation and calculations

Chromatograms were integrated and raltegravir concentrations were calculated with Empower 2.0 (Waters Software). Standard curve was constructed from linear least-squares regression was performed on the analyte to internal standard area ratio versus analyte concentration.

3. Validation of conditions

The method was validated according to guidelines for bioanalytical method validation [15,16]. Linearity of the method was assessed by analyzing seven complete standard curves (9 concentrations ranging from 10 to 10,000 ng/mL) on 7 separate days by linear regression with a weighting factor of $1/C$ where C is the analyte concentration. Intra- and inter-precision (coefficient of variation (CV%)) and accuracy (means, standard error) were evaluated using the 3 quality control samples. According to US

Table 1
Selectivity against drugs commonly used with raltegravir.

(a) Selectivity in vitro (retention time: if drug is retained by the column)	(b) Selectivity in vivo: drugs associated (27 patients)		
	ARV	Antibiotics, antiviral	Other PO administrated drugs
Zidovudine (26 min)	Darunavir	Ciprofloxacin	Clorazepate dipotassique
Lamivudine,	Ritonavir	Amoxicillin	Acepromazine
Didanosine (1.5 min)	Abacavir	Sulfamethoxazole	Aceprometazine
Indinavir (10 min)	Tenofovir	Trimethoprime	Perindopril
Saquinavir	Fosamprenavir	Levofloxacin	Metformine
Ritonavir	Lopinavir	Proguanil	Omeprazole
Nelfinavir + M8 metabolite	Nelfinavir	Atovaquone	Levoceterizine
Lopinavir	Etravirine	Ribavirine	Zolpidem
Atazanavir (6 min)	Atazanavir	Peginterferon alfa-2a	Citalopram
Amprenavir (40 min)	Emtricitabine		Hydroxyzine
Ritonavir	Nevirapine		Bromazepam
Efavirenz	Lamivudine		Mirtazipine
Nevirapine (3 min)	Indinavir		Racecadotril
Tipranavir	Maraviroc		Thiamine
Darunavir (40 min)	Enfuvirtide		
	Saquinavir		
	Zidovudine		

Food and Drug Administration regulations, <±15% error in statistical parameters is considered acceptable [15]. Lower limit of quantification (LOQ) was defined as the lowest concentration with both relative standard deviation (CV%) and percent deviation from the nominal concentration (% dev) were less than 20%.

The specificity of the method was evaluated by injecting solutions containing other antiretroviral drugs (all commercial protease inhibitors and non-nucleoside reverse transcriptase inhibitors) and commonly used medications (such as antibiotics) in the chromatographic system, with the analytical conditions used for raltegravir. Interference from endogenous compounds was investigated using 5 blank plasma samples.

Raltegravir stability was assessed in plasma samples aliquoted in different types of tubes (polyethylene and polypropylene) at ambient temperature, +4 °C and −20 °C for 24 and 72 h, to optimize plasma sample storage. Freeze–thaw stability of raltegravir was determined by assaying the three routine quality control samples in triplicate over three freeze-thawing cycles.

In order to assess an eventual interaction between vacutainer tubes used for blood collection, stability of raltegravir was also evaluated in whole blood collected in EDTA vacutainer tubes to determine the best conditions to collect raltegravir blood samples, focusing on the acceptable times between blood collection, centrifugation and plasma sample freezing. A pool of whole blood samples containing raltegravir was prepared and divided into 8 EDTA vacutainer tubes. These 8 samples were centrifuged and frozen at different times. The relative error was calculated for each sample, and the raltegravir concentration of the sample centrifuged and frozen at $t = 0$ h was taken as reference. The recovery of raltegravir following the extraction procedure was determined with 3 levels of quality control samples (75, 1500 and 7500 ng/mL) analyzed in triplicate.

4. Clinical sample analysis

To ensure the applicability of the method, clinical samples were assayed and results presented. Twenty-seven blood samples from patients treated with raltegravir (400 mg twice a day) were collected in sodium EDTA tubes and centrifuged at 3000 rpm for 10 min at 20 °C. Plasma was transferred to clean polypropylene tubes and stored at −20 °C until assay.

5. Results

5.1. Chromatographic characteristics

Representative chromatograms of raltegravir, extracted and analyzed under the conditions of the assay and of the LOQ (10 ng/mL), and a clinical sample are shown in Fig. 1 and demonstrate excellent separation of the raltegravir and IS, with short retention times around 10.3 and 12.4 min. Darunavir was eluted at a 40 min retention time and detected at 320 nm and that explained the 45 min run-time analysis. Neither endogenous substances, nor drugs listed in Table 1 interfered with raltegravir and IS at 320 nm. No interference with the glucuronide was observed with the PDA detection. This method was used in a clinical trial where patients received raltegravir co administrated with etravirine and darunavir, during the assays, no interference was shown with the plasma of these patients.

5.2. Calibration curve and linearity

Peak area ratios of raltegravir to IS for the calibration standards were proportional to the concentration of raltegravir in plasma over the range tested. As the variance increased in proportion to the concentration, the best weighting was $1/C$. The seven standard curves were linear from 10 to 10,000 ng/mL, with a mean equation $3.420C - 0.06888$ and an average coefficient of correlation of 0.9932.

5.3. Precision, accuracy and limit of quantification

Intra- and inter-assay precision and accuracy with 3 concentrations and the LOQ are reported in Table 2.

5.4. Extraction method and recovery

Mean (±SD) of raltegravir recovery (±SD) were 85.1% (±9.27%), 69.7% (±11.65%) and 84.4% (±7.67%), for human plasma raltegravir concentrations of 25, 500 and 10,000 ng/mL, respectively.

5.5. Stability of raltegravir

According to the relative biases (versus raltegravir concentration at $t = 0$ h) presented in Table 3, raltegravir is stable in human plasma for 24 h when the polypropylene tubes were stored at +4

Table 2
Precision and accuracy of RTG in human plasma.

	Theoretical RTG concentration (ng/mL)	Observed RTG concentration (ng/mL)	Relative error (%)	CV (%)
Within-day				
$n=6$	10	8.5	−15.0	12.3
	75	70.5	−6.0	3.1
	1500	1367.0	−8.9	3.7
	7500	7462.0	−0.5	5.5
Between-day				
$n=8$	75	69.9	−6.8	5.3
	1500	1457.6	−2.8	6.2
	7500	7255.0	−3.3	5.2

Table 3
Stability of raltegravir in human plasma at +4 °C, +25 °C in polyethylene and polypropylene tubes (bias (%) calculated from initial result of initial injection of QC samples).

Theoretical concentration (ng/mL)	Polypropylene tube				Polyethylene tube	
	24 h		72 h		24 h	
	+4 °C	+25 °C	+4 °C	+25 °C	+4 °C	-20 °C
75	−0.7%	−4.9%	−14.0%	−18.2%	−22.5%	−16.7%
1500	−4.8%	−6.8%	−9.2%	0.1%	−6.5%	−11.1%
7500	−5.5%	7.8%	−5.6%	−7.3%	−16.2%	−8.4%

and +25 °C. Raltegravir was not stable when stored in polyethylene tubes at +4 and +25 °C, even for 24 h.

Freeze–thaw stability biases ranged from 0.2 to 8.2%, from 0.1 to 1.6% and from 1.3 to 6.2% for the low, medium and high controls. Stability of raltegravir in whole blood samples stocked in EDTA vacutainer tubes was evaluated. All relative errors calculated for the samples were lower than 9.1%, as shown in Table 4.

5.6. Application of the method to patient samples

Plasma trough concentration of raltegravir was quantified in 27 patients receiving raltegravir 400 mg twice daily (samples collected in EDTA vacutainer tubes) with the described method. The method was successfully applied to these samples with plasma trough concentrations ranging from 14 to 2934 ng/mL (median 210 ng/mL).

6. Discussion

The aim of this work was to develop and validate an assay for raltegravir in human plasma, which would be suitable for clinical pharmacology purposes. Among the available analytical methods, HPLC with PDA detection appeared to be the method of choice because LCMSMS is still not widespread in hospital laboratories. For clinical pharmacology studies, a simple and rapid extraction step is necessary, which is why liquid–liquid extraction was chosen. This extraction method was also used by Long et al. [9], Merschman et al. [7] but with higher limit of quantification and by Poirier et al. [11] who used spectrofluorometric detection. A solid-phase extraction of 90% efficiency was proposed by Rezk et al. [12] and Notari et al. [14], but it is more expensive than liquid–liquid extraction.

Table 4
Stability of raltegravir in all-blood samples stocked in vacutainer® EDTA tubes.

	Target concentration of RTG		
	2340 ng/mL		
Centrifugation time delay (h)	0	8	24
Freezing time delay (h)	24	8	24
Mean concentration (ng/mL)	2553	2377	2425
Relative error	9.1%	1.6%	3.6%

Several columns were tested to assay raltegravir, such as Lichrosphere 100 RP 18e (125 mm × 4 mm, 5 μm); Zorbax® phenyl Interchim (4.6 mm × 250 mm, 5 μm); Lichrosphere CN (250 mm × 4.6 mm, 5 μm), and Satisfaction C8 Plus (250 mm × 3 mm, 5 μm), but retention times of raltegravir which is a hydrophilic compound were too short (<5 min) and raltegravir peak tailing, even with a buffered mobile phase or when triethylamine was added. A dC18 column (Atlantis dC18 3 μm) specific to hydrophilic components such as raltegravir (D_{ow} = 2.80 at pH 7.4) [17] was evaluated and found to provide adequate peak shape and resolution for the analysis of raltegravir.

Several mobile phases were tested with the chosen column: water/acetonitrile mixture that was associated with a bad peak resolution, and acetate buffer/acetonitrile in different proportions, leading to the chosen one (71%/29%) that provided a retention time superior to 10 min. Ionic strength of acetate buffer solution (pH 4, 0.1 mol/L concentration and 0.1 mol/L ionic strength) is needed to maintain raltegravir as a non-ionised compound, and ensure symmetry and thickness of raltegravir peak. Quinoxaline was chosen as an internal standard for its sufficient UV absorbance at 320 nm, its capacity of extraction, and its selectivity, even though its molecular structure is different from raltegravir.

The LOQ found with our method (10 ng/mL) is higher than the LOQ of other quantification methods known for their greater sensitivity, such as LCMSMS [4,7,8,10,18], but greater than other UV detection quantification methods [12–14]. Limit of quantification of the 3 quantification methods using HPLC with UV detection were: 20 ng/mL with 100 μL plasma sample and a solid/liquid extraction step for Rezk et al. [12]; 23.4 ng/mL with a 500 μL plasma sample and a solid/liquid extraction step for D'avolio et al. [13], and 19.5 ng/mL with a 600 μL plasma sample and a solid/liquid extraction step for Notari et al. [14]. A quantification method using HPLC with fluorimetric detection gave an LOQ of 2.5 ng/mL using a larger plasma sample volume than our method (500 μL versus 300 μL) [11]. However, the LOQ of 10 ng/mL is sufficient to quantify plasma raltegravir trough concentration in patients according to previously published concentrations of raltegravir in healthy volunteers [19] who had a mean steady-state plasma trough concentration (range) of 200.6 nM (40–400 nM) [97 ng/mL (20–200 ng/mL)]. This assay was also successfully used to quantify raltegravir in plasma of HIV-infected patients. In this study, plasma raltegravir

concentration ranged from 14 to 2935 ng/mL. Of note, two of our 27 patients received raltegravir combined with atazanavir (400 mg once daily) a known inhibitor of UGT1A1 involved in raltegravir metabolism and therefore had higher plasma raltegravir trough concentration (2239 and 2935 ng/mL) [20]. Excluding those two patient samples, the median (range) trough concentrations of raltegravir was 210 ng/mL with large interindividual variability (18–1264 ng/mL), which is close to plasma raltegravir trough concentrations measured in healthy volunteers receiving raltegravir without atazanavir. Linearity was demonstrated up to 10,000 ng/mL which should enable assay of maximal concentrations [19]. In addition to stability studies performed by others, stability of raltegravir was studied in several types of tubes. Importantly, our results show that polyethylene tubes cannot be used, and raltegravir is stable in polypropylene tubes stored at −20 °C, and in whole blood samples in vacutainer ETDA tubes stored at ambient temperature for 24 h.

In conclusion, this accurate, specific and highly reproducible HPLC assay of raltegravir in plasma was validated. This assay can be applied to clinical pharmacology research in any analytical laboratory possessing HPLC with a PDA detector.

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