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Research Paper

A sensitive liquid chromatography coupled with mass spectrometry method for the intracellular and plasma quantification of raltegravir after solid-phase extraction

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Abstract

Introduction Liquid chromatography coupled with mass spectrometry for the quantification of raltegravir in human plasma and peripheral blood mononuclear cells has been developed.
Methods Sample preparations were based on a fully automated solid-phase extraction process. Mass spectrometric data were acquired in a single-ion monitoring method. Raltegravir and quinoxaline, the internal standard, were well separated in a gradient mode over 15 min.
Key findings Validation study exhibited excellent linearity, with good intra- and inter-day precision and accuracy.
Conclusions The assay was successfully applied to the raltegravir quantification in HIV-infected patients.
Keywords cellular concentration; HPLC-MS; raltegravir; solid phase extraction

Introduction

Raltegravir (MK-0518, Isentress) is the first commercially available antiretroviral agent targeting human immunodeficiency virus-1 (HIV-1) integrase, one of the three enzymes that play a crucial role in viral replication. Raltegravir is indicated, in association with other antiretrovirals, in the treatment of human acquired immunodeficiency syndrome (AIDS) in antiretroviral treatment-experienced or naïve adult patients with viral resistance.[1]

Very little data is available on the intracellular pharmacokinetics of raltegravir.[2,3] As HIV replicates within the cells, raltegravir must penetrate intracellularly at a concentration sufficient to inhibit viral replication. Consequently, monitoring intracellular drug concentrations is useful to assess raltegravir's cellular penetration. Several high-performance liquid chromatography (HPLC) methods using UV detection,[4] fluorescence detection[2,5] or tandem mass spectrometry (MS) detection[6–8] for raltegravir quantification in plasma have been previously reported, while only two studies have described intracellular quantification using HPLC-MS/MS.[9,10]

In this context, we developed and validated a simple, sensitive and selective assay for quantification of raltegravir in two matrices, peripheral blood mononuclear cells (PBMCs) and plasma, using liquid chromatography coupled with a single quadrupole mass spectrometer (LC-MS). This method is innovative in the pretreatment of samples. In the literature, no method describes the extraction of raltegravir from PBMCs and plasma using the solid-phase extraction (SPE) technique which presents many advantages. Having developed this method, we can provide initial results on the intracellular penetration of raltegravir.

Materials and Methods

Chemicals and reagents

Raltegravir monopotassium salt and quinoxaline, used as the internal standard (IS) for this assay, were obtained from Merck (Rahway, USA). All chemicals were analytical reagent grade and all solvents were of HPLC grade. Acetonitrile and methanol were purchased from Scharlau (Barcelona, Spain). Sterile distilled water was obtained from Aguettant (Lyon,

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France). Ultra-pure water was obtained using a water purification system. Ammonium hydroxide, sodium azide, formic acid and ammonium were provided by Prolabo (Fontenay-sous-Bois, France). Vacutainer cell preparation tubes (CPTs) (Becton Dickinson, Le Pont-de-Claix, France) were used for the separation of PBMCs from whole blood. KOVA glasstic slides were provided by Hycor Biomedical Inc. (Garden Grove, USA). To validate the method in human plasma, we used six different batches of pooled drug-free plasma from healthy volunteers (French Blood Agency). These plasma pools were portioned and then frozen at −20°C. The cell matrix for validation of the assay was Jurkat cells. The cells were prepared as previously described to obtain a cell suspension density at 3×10^6 cells/ml.[10]

Equipment

An LC system (1100 series system; Agilent Technologies, Palo Alto, USA) coupled with a single quadrupole 1100 mass spectrometer, and data-acquisition station to control the LC-MS system (1956B; Agilent Technologies) were used. Mass spectral analyses were fitted with an atmospheric pressure chemical ionization (APCI) source and operated in the positive ionization mode. Discharge current was fixed at 5 µA and capillary voltage at 3800 V. Capillary temperature was maintained at 300°C. The nebulizer gas was nitrogen at a flow rate of 6 L/min and nebulizer pressure was 60 psig. Elution was performed using a Prontosil C18 AQ+ column (150 mm × 4.6 mm, 5 µm particle size; Bischoff Chromatography, Germany). The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% formic acid in water). Chromatographic separation was performed with a quick stepwise gradient at a flow-rate of 0.8 ml/min at 20°C (Table 1). Chromatographic run time was 15 min.

A Jouan GR4-22 centrifuge (Saint Herblain, France) was used for centrifugation during sample preparation.

An automated SPE method using OASIS MAX extraction cartridges (Waters, USA) was performed on an ASPEC Xli system (Gilson Medical Electronics France, Villiers de Bel, France).

Preparation of calibration standards and quality control samples

A stock solution of raltegravir (10 µg/ml) was prepared by appropriate dissolution of powders in plasma and stored at −20°C. A solution of quinoxaline at 1 µg/ml was prepared in water and used as the internal standard solution. Stock solutions of raltegravir were diluted in drug-free human plasma to obtain calibration standards containing 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml raltegravir. Concentrations of quality control (QC) samples used for the determination of total components were 75, 750 and 3000 ng/ml raltegravir and QCs were prepared from different stock solutions (15 µg/ml) to the calibration standards.

Calibrators used for the intracellular quantification of raltegravir were prepared in Na_2HPO_4 buffer (pH 7.4) to obtain calibration standards of 2.5, 5, 10, 50, 100, 250 and 500 ng/ml and 50 µl of each calibrator was loaded into Jurkat cells. Quality control samples were prepared in the same buffer to obtain 7.5, 75 and 300 ng/ml raltegravir.

Table 1 Gradient of mobile phase

Time (min)	Acetonitrile (%)	Formic acid 0.1% (%)
0.3–5	40	60
3.5–8	60	40
8–11	80	20
11–12	60	40
12–15	40	60

Sample collection

Clinical samples were collected from HIV-infected patients undergoing high active antiretroviral therapy (HAART) including raltegravir. Approximately 8 ml of peripheral venous blood was collected into Vacutainer CPTs containing sodium citrate as anticoagulant and stored at 4°C before initial centrifugation. PMBCs were isolated by density gradient separation and worked in 30 min maximum after collection in order to measure the real quantity of raltegravir at sampling time. Vacutainer CPTs were centrifuged at $1650 \times g$ for 20 min at 20°C. Cellular fraction was transferred to a fresh tube and centrifuged at $600 \times g$ for 10 min at 4°C. The pelleted PBMCs were washed with 2 ml ice-cold PBS, put on KOVA slide for cell count and centrifuged as described above. The cell pellet was suspended in 200 µl of a solution of α1-acid glycoprotein 1 mg/ml in sodium azide (0.1%, w/v). Cell lysates were stored at −80°C until analysis by LC/MS assay.[11]

Solid-phase extraction method

One hundred microlitres of the IS (1 µg/ml) and 750 µl of 2% NH_4OH were added to 200 µl of calibrator, QC or patient samples. Then, samples were extracted by the automated solid-phase process. The cartridges were primed with 1 ml of methanol and 1 ml of distilled water. Pretreated plasma and PBMC samples (1 ml) were loaded into the cartridges. After a washing step with 1 ml of 1% NH_4OH and 1 ml of MeOH/H_2O (50/50, v/v), the analytes were eluted with 2 ml of methanol containing 2% of formic acid. The solvent was evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 100 µl of the mobile phase acetonitrile/formic acid (35/65; v/v) and 20 µl were injected into the chromatographic system. The intracellular drug concentration was calculated from a measured volume of 0.4 pl for each PBMC.[12]

Validation studies

Validation of the assay was based on FDA guidelines for bioanalytical method validation.[13]

Linearity

Linearity was assessed using daily calibration curves consisting of a blank sample and seven calibrator concentrations (range: 2.5–500 ng/ml in PBMCs and 50–5000 ng/ml in plasma). Daily calibration curves were constructed using the ratios of the observed peak areas of raltegravir to the IS. Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ where y was the peak area ratio, x the concentration of raltegravir, m and b respectively the slope and intercept of the curve. Unknown concentrations were

computed from the linear regression equation of the peak area ratio against concentration for the calibration curve.

Limits of detection and quantitation

The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was defined by the minimum amount that gave precise measurements (accuracy and precision both less than 20%) and was determined by injecting five times a number of spiked samples with decreasing concentration of the analyte.[13]

Precision and accuracy

Accuracy was assessed as the percentage bias from the nominal concentration (% bias), and precision was calculated as the relative standard deviation (RSD). The intra-day accuracy and precision of the method were estimated from the back-calculated concentrations from QC samples. Six replicates of each QC level (7.5, 75 and 300 ng/ml for PBMCs; 75, 750 and 3000 ng/ml for plasma) were processed and injected on four separate days to determine inter-day variability. The acceptable intra- and inter-day precision and % bias were set at $\leq$15%.

Recovery

Extraction recovery was determined by comparing the analytical results of extracted samples at different QC ($n = 3$) concentrations with pure standards at the same concentration without extraction.

Specificity, selectivity and matrix effect

To assess the specificity-selectivity of the method, we analysed six different batches of pooled human plasma and PBMCs without the IS. The interference from endogenous compounds was examined at the retention times of raltegravir and IS. Potential interference by other antiretroviral agents concomitantly administered to the patients was evaluated by spiking blank PBMCs and human plasma with them (darunavir, lopinavir, atazanavir, efavirenz, etravirine and emtricitabine). No other concomitant drugs were investigated.

Undetected matrix components co-eluting with analytes may adversely affect the reproducibility of analyte ionization in the APCI source of the spectrometer. To verify the absence of ion-suppression or ion-enhancement effects attributable to the matrix, the following experiments were performed. Three replicates of each blank human plasma ($n = 3$) and PBMC ($n = 3$) sample were subjected to SPE. The final dried extracts were reconstituted with 100 µl of mobile phase containing raltegravir at three QC concentrations or the IS, separately. The corresponding areas were then compared with those of the standard solutions prepared in the mobile phase at equivalent concentrations. The result of the matrix effect was calculated as $100 \times (A_{st} - A_{extr})/A_{st}$, where A_{extr} is the peak of raltegravir or IS from the post-extraction spiked sample (extracted blank plasma or PBMC sample spiked with raltegravir or IS), while A_{st} is the peak area of raltegravir and IS from direct injection of the standard solution. An interval of 85–115% was considered acceptable.

Dilution effect and memory effect

The dilution effect of plasma and PBMC samples was validated with a high QC diluted to half, and analysed six times. Memory effect was tested by injecting three blank plasma and three blank PBMCs after the highest QC and the percentage of disappearance of raltegravir was evaluated.

Stability

The stability of raltegravir in plasma and PBMCs was studied under the following conditions: (1) room temperature, (2) freeze–thaw cycles, and (3) long-term storage at −20°C. For each stability determination, QC samples spiked at 75 and 3000 ng/ml in plasma and 7.5 and 300 ng/ml in PBMCs were assayed in triplicate.

Statistical analysis

Concentrations were expressed as medians and interquartile ranges [Q1; Q3]. Statistical analysis of intracellular and plasma concentrations of raltegravir was performed using the Spearman correlation test. Data was analysed with Statistica software (version6.1; StatSoft, Créteil, France). $P < 0.05$ defined statistical significance. Inter-patient coefficient of variation (CV %) was determined for plasma and PBMCs using the formula (standard deviation/mean of concentrations) × 100.

Application to clinical study

Study population

The study was approved by our local Ethics Committee (CHU de Bordeaux). Patients gave informed written consent before being included. The patients included in this study were HIV-1-infected patients followed up at the Bordeaux University Hospital within the Aquitaine Cohort of the Groupe d'Epidémiologie Clinique du SIDA en Aquitaine (GECSA). The Aquitaine Cohort is a prospective hospital-based cohort of HIV-1-infected patients under routine clinical management, initiated in 1987 in the Bordeaux University Hospital and four other public hospitals in Aquitaine, south-western France.[14] Clinical, biological and therapeutic information were collected prospectively at each visit. Seventeen pretreated HIV-infected patients (12 men and 5 women, mean age 49 years) were included in this cohort and followed in this observational study because they started a raltegravir (400 mg twice daily)-containing HAART. Patients gave informed written consent before being included.

Raltegravir quantification results

Intracellular and plasma raltegravir concentrations 3 h post-dose (C_{max}) and 12 h post-dose (C_{min}) were measured. Intracellular concentrations were determined in PBMCs. Raltegravir was quantified using the validated HPLC assay coupled with mass spectrometry.

Results

Here, we describe a simple LC-MS assay for the determination of plasma and intracellular raltegravir concentrations. We optimised the various experimental variables, including mass spectrometry conditions and the nature and composition of mobile phase.

Chromatographic and mass spectrometry characteristics

Under the specified chromatographic conditions, typical retention times of raltegravir and IS (quinoxaline) were 6.7

Table 2 Intra-day and inter-day accuracy and precision of raltegravir determination in peripheral blood mononuclear cells and plasma

	Nominal amount (ng/ml)	Mean measured (ng/ml)	Accuracy (%)	Relative standard deviation (RSD %)	*n*
PBMCs					
Intra-assay	7.5	7.31	103.11	13.28	6
	75	74.02	99.47	6.67	6
	300	310.48	106.48	2.09	6
Inter-assay	7.5	7.69	102.59	12.39	6
	75	71.44	95.25	8.69	6
	300	309.17	103.06	4.39	6
Plasma					
Intra-assay	75	64	84.67	11.56	6
	750	761	101.44	2.26	6
	3000	3066	102.19	4.46	6
Inter-assay	75	64	98.19	8.21	6
	750	7553	97.55	4.91	6
	3000	3156	97.37	5.15	6

PBMCs, peripheral blood mononuclear cells.

and 7.5 min, respectively. Selected ions for monitoring (m/z) were 445.4 for the protonated adduct ion of raltegravir and 314.2 for quinoxaline, respectively.

Performance characteristics

Linearity

The assay was linear over the range 2.5–500 ng/ml in PBMC matrices and 50–5000 ng/ml in plasma and showed good correlation coefficients ($r^2 > 0.99$ for both matrices), with regression intercepts not statistically different from zero.

Limit of detection and quantitation

For raltegravir, the LOD and LOQ were determined, respectively, as 1 ng/ml and 2.5 ng/ml.

Precision and accuracy

The results of the precision and accuracy experiments are given in Table 2. Intra-assay and inter-assay accuracy varied from 99.47% to 106.48% and from 95.25% to 103.06%, respectively, for PBMCs. Intra-assay and inter-assay accuracy varied from 84.67% to 102.19% and from 97.37% to 98.19%, respectively, for plasma. Within-day precision varied from 2.09% to 13.28% and from 2.26% to 11.56% for PBMCs and plasma, respectively, and between-day precision varied from 4.39% to 12.39% and from 4.91% to 8.21% for PBMCs and plasma, respectively. The results indicated that the method was accurate and precise in the calibration range of each matrix.

Recovery

Multiple samples ($n = 6$) at each of three different concentrations (low, medium, high) were assayed. The recoveries are shown in Table 3. The mean extraction recovery was 88.5% in PBMCs and 91.1% in plasma. Precision was within 15% for both matrices.

Specificity, selectivity and matrix effect

No peaks from endogenous compounds were observed at raltegravir selected transition and retention time in blank PBMC and plasma extracts. The product ion monitoring was chosen, given its relative abundance, while avoiding possible structural analogies with others drugs. The assay did not show any significant interference from other antiretroviral drugs taken at therapeutic dosages by patients.

Table 3 Recovery determination of raltegravir in human peripheral blood mononuclear cells and plasma ($n = 6$)

	Nominal amount (ng/ml)	Recovery (%)	Relative standard deviation (RSD %)	*n*
PBMCs	7.5	88.2	11.84	6
	75	89.2	13.43	6
	300	88.0	14.07	6
Plasma	75	89.4	9.44	6
	750	92.3	10.71	6
	3000	91.6	11.03	6

PBMCs, peripheral blood mononuclear cells.

During experiments, no difference in signal of either raltegravir or IS at their respective retention times was observed when a plasma or a PBMC extract was injected. The result of matrix effect following the SPE process ranged from 87 to 111%, so that the matrix effects were minimal. Therefore, ion-suppression or ion-enhancement was considered not to influence the analysis.

Dilution effect and memory effect

Quantification of half-diluted QC gave reliable results: 103.3% for accuracy and 5.91% for RSD. No memory effect was detected after injection of the highest QC – raltegravir was not quantifiable in the three blanks.

Stability

QC samples were left standing at ambient conditions for 24 h before analysis against a freshly prepared standard curve. The results in Table 4 indicate that raltegravir is stable in plasma and PBMC for at least 24 h at room temperature.

Table 4 Stability of raltegravir in peripheral blood mononuclear cells and human plasma under different storage conditions

Description	PBMCs					Plasma				
	Low QC (7.5 ng/ml)		*n*	High QC (300 ng/ml)		*n*	Low QC (75 ng/ml)		*n*	High QC (3000 ng/ml)		*n*
	Mean predicted concentration ± SD	RSD (%)		Mean predicted concentration ± SD	RSD[a] (%)		Mean predicted concentration ± SD	RSD[a] (%)		Mean predicted concentration ± SD	RSD[a] (%)	
Room temperature (24 h)	7.18 ± 0.07	1.08	3	281.3 ± 14.9	5.32	3	72.6 ± 0.5	0.76	3	2943 ± 57	1.97	3
Freeze–thaw (3 cycles)	7.47 ± 0.09	1.33	3	294.6 ± 8.5	2.89	3	72.9 ± 0.9	1.36	3	2980 ± 56	1.88	3
Long-term storage	7.61 ± 0.1	1.39	3	306 ± 9.3	3.03	3	73.9 ± 0.8	1.09	3	3024 ± 69	2.11	3

PBMCs, peripheral blood mononuclear cells; QC, quality control; RSD, relative standard deviation.

Table 5 Plasma and PBMC concentrations of raltegravir in HIV-infected patients ($n = 18$)

	C_{min} (ng/ml)[a]	C_{max} (ng/ml)[a]
Plasma	300 [295; 315]	900 [600; 1100]
PBMCs	53 [45.2.5; 78.1]	94.1 [46.1; 102.6]

[a]Values are expressed as medians [Q1; Q3]. PBMCs, peripheral blood mononuclear cells. C_{min}, concentration measured 12 h post-dose; C_{max}, concentration measured 3 h post-dose.

QC samples were subjected to three freeze–thaw cycles. Each freeze–thaw cycle consisted of keeping the samples frozen for a minimum of 12 h. After the third freeze–thaw cycle, the samples were analysed against a freshly prepared standard curve. The results in Table 4 indicate that raltegravir is stable in plasma and PBMCs through at least three freeze–thaw cycles.

QC samples were stored at −20°C. After three months, the samples were analysed against a freshly prepared standard curve. The results in Table 4 indicate that raltegravir is stable in plasma and PBMCs for at least three months at −20°C.

Determination of intracellular and plasma raltegravir concentrations

Median [Q1; Q3] raltegravir C_{max} and C_{min} were 900 ng/ml [600; 1100] and 300 ng/ml [295; 315] in plasma and 94.1 ng/ml [46.1; 102.6] and 53 ng/ml [45.2; 78.1] in PBMCs, respectively (Table 5). The median intracellular concentration : plasma concentration ratio was approximately 13%. Correlation was observed between intracellular and plasma concentrations ($\rho = 0.66$, $P = 0.05$). High inter-patient variability of intracellular and plasma raltegravir concentrations were noted (inter-patient CV % was >40% for both matrices).

Discussion

Raltegravir is the first-in-class HIV integrase inhibitor to become available for use in both naive and antiretroviral-experienced patients. Due to its unique mechanism of action, rapid and potent antiviral effect, and excellent safety and tolerability profile, this new drug is likely to become extensively used and considerable research remains to be completed. Currently, there is very little data on intracellular raltegravir penetration. Previously, two assays using mass spectrometry have been developed.[9,10] These used a protein precipitation extraction process. However, no intracellular data was presented in these two studies. Ter Heine *et al*. conducted a pharmacokinetic study of raltegravir in heavily pretreated patients, but intracellular raltegravir concentrations could not be detected.[9] In this context, we aimed to develop and validate a simple and sensitive LC-MS bioanalytical method allowing quantification of raltegravir in plasma and cells of HIV-infected patients, and apply it to a pharmacokinetic investigation.

Compared with published methods,[2,3,5–10,12] we developed a solid-phase extraction method to minimize quantitative errors resulting from ion suppression or ion enhancement due to the matrix. Thus, we used a new liquid-solid fully

automated extraction procedure for the preparation of samples. This enabled us to increase selectivity of the assay and so reduce the matrix effect. Moreover the advantage of the SPE method is its automated process for managing injection of the IS in samples, the dilution of samples and the elution of analytes allowing good reproducible data. We chose Oasis MAX cartridges, which provided the best results of recovery and specificity with a satisfactory accuracy, precision and reproducibility.

Regarding the metabolites, raltegravir is metabolized into glucuronide G-RAL which might interfere in the dosage method but since detection occurs in LC-MS and the m/z G-RAL (621) ratio is different from the m/z raltegravir (445,4) ratio, the results that we present indeed concern raltegravir and not the total form raltegravir + G-RAL.

A limit of the intracellular assay is the difficulty in assessing the PBMCs' drug efflux before their treatment. We applied two processes to minimize raltegravir efflux: PBMC handling time (which must be as short as possible) and initial storage of CPTs harvested at +4°C before proceeding with plasma/cell separation. Given the mechanism of action of raltegravir, the percentage of effluxed drug remains low because raltegravir, once it penetrates the cell, will bind to its target (integrase of nucleus) and also it is not the substrate for efflux proteins of the multidrug resistance type which are, moreover, inhibited at +4°C. We cannot say that there is no efflux of raltegravir but, given the operating conditions and the pharmacodynamics, this efflux seems negligible.

Finally, the method was applied to determine intracellular and plasma levels of raltegravir in HIV-1-infected patients. Observed raltegravir plasma concentrations were comparable with those previously described.[2,12] To our knowledge, two studies reported intracellular concentrations of raltegravir[2,3] where the results are consistent with ours. We also observed a low raltegravir intracellular PBMC accumulation (average 13% of plasma concentration) with a large variability in concentrations, probably reflecting variability in expression of cellular mechanisms (drug efflux, binding) and metabolizing enzymes, similarly to other antiretrovirals such as efavirenz.[15] This data was recently confirmed for raltegravir.[3] Knowing that there is a positive correlation between the plasma and intracellular concentrations of raltegravir, the plasma concentration may be sufficient to provide therapeutic drug monitoring, as intracellular determination is not accessible for all laboratories. This data was confirmed by Fayet Mello *et al*.[3]

Conclusion

In conclusion, a simple, accurate and specific method for quantification of raltegravir in plasma and PBMCs has been developed and validated using LC-MS and, for the first time, an automated and reproducible SPE method. With this new method for raltegravir intracellular and plasma quantification, we confirmed the low raltegravir PBMC penetration with correlated plasma and intracellular concentrations despite the large variability of these concentrations. Other pharmacokinetic studies in larger cohorts need to be conducted to confirm these initial results.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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