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Short communication

Quantification of the HIV-integrase inhibitor raltegravir (MK-0518) in human plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A simple and sensitive HLPC method with fluorescence detection was developed for the accurate determination of the first licensed HIV integrase inhibitor raltegravir in human plasma. A 500-μL plasma sample was spiked with delavirdine as internal standard and subjected to liquid–liquid extraction based on a previously described assay i.e. using hexane/methylene chloride (1:1, v/v%) at pH 4.0. HPLC was performed using a Symmetry Shield RP18 column (150 mm × 4.6 mm), a gradient elution of acetonitrile –0.01% (v/v) triethylamine in water adjusted to pH 3.0 at a flow rate of 1 mL/min and a fluorimetric detector set at 299 and 396 nm as excitation and emission wavelengths, respectively. The retention time was 5.0 min for internal standard and 6.4 min for raltegravir. Calibration curves were linear in the range 5–1000 ng/mL and the accuracy of quality control samples in the range 10–750 ng/mL varied from 98.3 to 99.1% and 98.3 to 101.0% of the nominal concentrations for intra-day and day-to-day analysis, respectively with a precision of 6.3% or less. Among the other antiretroviral drugs which can be given in association to HIV-infected patients, none was found to interfere with internal standard or raltegravir. The described assay was developed for the purpose of therapeutic drug of this HIV integrase inhibitor.

1. Introduction

Several studies suggest that therapeutic drug monitoring (TDM) of antiretroviral drugs may contribute to the clinical outcome of HIV-infected patients [1–6]. Even in daily practice the use of TDM remains controversial [7,8] it may be a valuable tool particularly in patients with limited treatment options.

Raltegravir (MK-0518, Isentress®) is the first FDA approved HIV-1 agent of a new pharmacological class called integrase inhibitors for treatment of experienced HIV-infected patients in combination with other antiretroviral agents. This drug acts by inhibiting the insertion of HIV DNA into the genome of target cells [9,10] and then preventing viral replication.

After oral administration, raltegravir is eliminated primarily by glucuronidation in the liver and exhibits a linear pharmacokinetic profile over a dose range 100–1600 mg with an apparent terminal half-life of about 9 h. Its pharmacokinetics display marked variability and at 400 mg twice daily inter- and intra-patient of its trough plasma concentrations were 212 and 122%, respectively [11]. Due to this considerable variability of plasma concentrations, raltegravir therapeutic drug monitoring could be a valuable tool to ensure effective exposure to the drug.

An HPLC–MS/MS method was developed for the accurate determination of raltegravir in human plasma [12] to support clinical studies but this analytical equipment is not available in all laboratories. We present an HPLC assay of this antiretroviral using a fluorescence detection for purpose of raltegravir therapeutic monitoring in HIV-infected patients.

2. Experimental

2.1. Chemicals and reagents

Raltegravir (Fig. 1) was a gift of Merck Research Laboratories (Rahway, NJ, USA) and delavirdine used as internal standard was obtained from Agouron Pharmaceuticals (San Diego, CA, USA). Acetonitrile, triethylamine and water were of HPLC quality grade and purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Hexane, methylene chloride, acetic acid, 85% phosphoric acid, sodium acetate were of analytical-grade and obtained from VWR (Fontenay-sous-Bois, France).

2.2. Instrumentation

The HPLC unit consisted of a Waters (Saint Quentin en Yvelines, France) 600E multisolvent delivery system, a 2475 multi wave-

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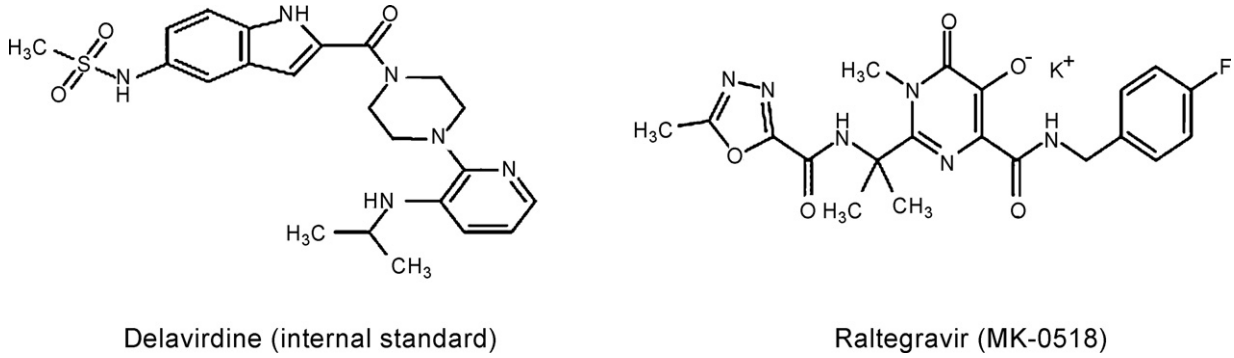

Fig. 1. Chemical structures of raltegravir and the internal standard delavirine.

lengths fluorescence detector coupled to the Empower Pro data acquisition software (version 2.0) and a WISP 717 plus automatic injector thermostated at 4 °C. The column was kept at 25 °C (oven Jetstream 2, Jasco, Nantes, France).

2.3. Chromatographic conditions

Separations were performed at on a Symmetry Shield 3.5 μm RP18 column (15 cm × 4.6 mm i.d.) equipped with a 2-cm precolumn packed with the same material (Waters). The gradient mobile phase consisted of acetonitrile (A) −0.01% (v/v) triethylamine in water adjusted to pH 3.0 with 85% phosphoric acid (B). The starting mobile phase consisted of 40% A–60% B (v/v) switched to 75% A–25% B at 2 min and at 13 min the system was re-equilibrated with the initial conditions. The run-time was 21 min at a flow rate of 1 mL/min.

The fluorescence detector was set at 299 and 396 nm as excitation and emission wavelengths, respectively.

2.4. Preparation of standard solutions and standard curves

A 1 mg/mL weighted stock solutions of raltegravir base (molecular weight of raltegravir monopotassium salt = 482.51 g/mol and molecular weight of raltegravir base = 443.41 g/mol) and delavirdine (internal standard) were prepared in methanol. The stock solution of raltegravir was diluted in methanol/water (1:1, v/v%) to obtain working standard solutions ranging from 100 to 20,000 ng/mL. The working solution of internal standard was a 1/25 dilution of its stock solution in methanol/water (1:1).

Plasma standards were prepared by spiking 25 μL of working standard solution and 25 μL of internal standard working solution into 500 μL of human drug-free plasma to give raltegravir plasma concentrations of 5, 25, 100, 250, 500, 750 and 1000 ng/mL containing 1000 ng internal standard.

2.5. Preparation of plasma quality control samples

An independent 1 mg/mL stock solution of raltegravir base was prepared in methanol and appropriate working solutions in methanol/water were done to produce 1, 5 and 75 μg/mL standard control solutions. Plasma control samples at 10 (low QC), 50 (medium) and 750 (high) ng/mL were prepared by transferring 500 μL of these control solutions into 50-mL volumetric flasks filled with human drug-free plasma. These plasma quality controls were then aliquoted and stored at −20 °C.

2.6. Sample preparation

Into 10-mL glass tubes were added 500 μL of human plasma sample, 25 μL of working internal standard solution, 25 μL of methanol–water (1:1, v/v%) to compensate for the volume of working standard solutions used, 500 μL of 0.2 M pH 4.0 acetate buffer and 5 mL hexane/methylene chloride (1:1, v/v%). The tubes were capped and shaken horizontally for 10 min then centrifuged at 3000 rpm for 5 min at 4 °C. The organic layer was evaporated under air at 40 °C and the residue redissolved in 200 μL of the starting mobile phase (acetonitrile – pH 3.0, 40–60%, v/v%). A 100 μL-aliquot was injected into the HPLC system.

2.7. External quality control for antiretroviral drugs

The French Asqualab (Broussais Hospital, Paris) external quality control program for antiretroviral drugs and containing all the licensed HIV-protease inhibitors and non-nucleoside transcriptase inhibitors propose to analyze four times a year two unknown lyophilized human plasma samples containing these antiretrovirals at concentrations close to their trough and maximal concentrations usually observed at standard dosing regimen.

3. Method development

3.1. Chromatographic conditions

A symmetry shield column was previously used in the laboratory for the simultaneous determination of HIV-protease inhibitors and non-nucleoside transcriptase inhibitors and was tested for raltegravir analysis in binary acetonitrile–0.01% (v/v) triethylamine pH adjusted aqueous mobile phases. Peak shape and internal standard retention time were dependent on pH and pH 3.0 was found to be a good compromise between these parameters.

We found that raltegravir exhibited fluorescence properties. Fluorescence spectrum showed excitation and emission maximal wavelengths of 299 and 396 nm, respectively after injection of raltegravir in the starting mobile phase. When the fluorescence detector was coupled to a 2996 photodiode array detector (Waters) set at 210 nm and 25 ng raltegravir injected, drug peak-heights were 121,607 μV (fluorescence) and 10,156 μV (UV 210 nm). These raltegravir peak-heights correspond to signal to noise ratios of 585.1 and 26.6 for fluorescence and UV detectors, respectively. Therefore, fluorescence allows a more sensitive detection method for raltegravir determination rather than UV.

During the development of the method we found a late plasma endogenous peak (retention time: 17.9 min). The use of a mobile phase containing a high percentage of acetonitrile (75/25, v/v%)

during the run was necessary to eliminate this unwanted compound. Under these conditions no interfering peak was present during the analysis of subsequent samples. After that, chromatograms were only recorded during the first 8 min of the analytical run.

3.2. Extraction conditions

The extraction procedure was based on conditions previously described [12] i.e. plasma samples buffered at pH 4.0 using a 1:1 (v/v%) solvent mixture of hexane and methylene chloride.

3.3. Stability of raltegravir standards and plasma samples

The stability of raltegravir was previously fully studied [12] and was not tested. It was found that stock raltegravir was stable after 8-month of storage. Plasma raltegravir samples are stable at room temperature for up to 5 h, for at least 23 months when stored at −20 °C and after 3 freeze–thaw cycles.

4. Results and discussion

4.1. Internal standard

Among the different drugs tested as internal standard, only delavirdine, an HIV non-nucleoside transcriptase inhibitor, was found to have fluorescence wavelengths closed to raltegravir, a short retention time and a reproducible coefficient of extraction. This antiretroviral is not used in France and infrequently used in other countries due to its three times daily dosing-limitation and the lack of recent clinical studies with this drug. Therefore, delavirdine is not recommended as initial therapy or for the treatment of experienced HIV-infected patients [13].

4.2. Representative chromatograms

Under the described chromatographic conditions, retention times of internal standard (delavirdine) and raltegravir were 5.0 and 6.4 min, respectively. Assays performed on drug-free human

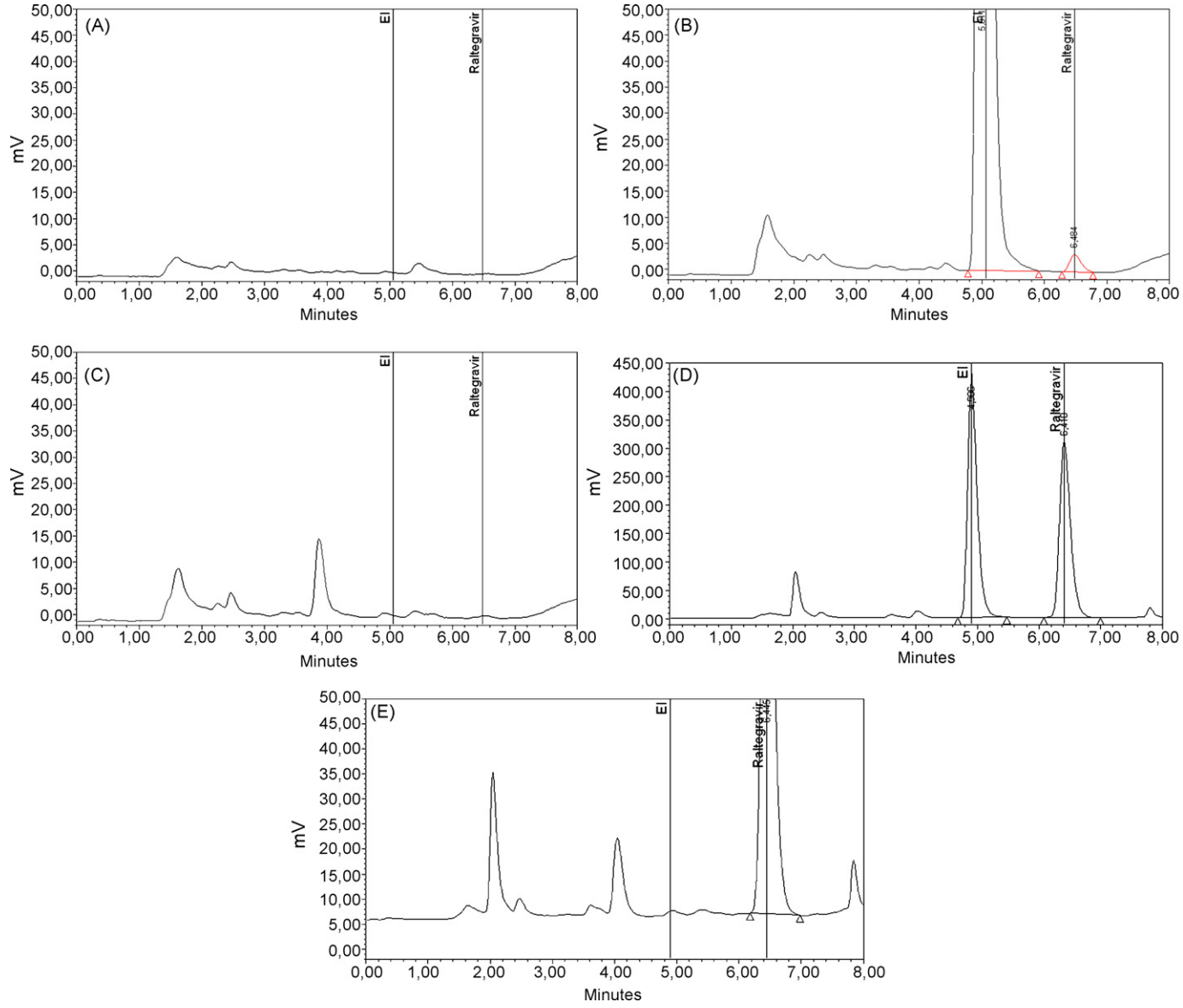

Fig. 2. Representative chromatograms of a blank plasma sample (A), a 5 ng/mL standard containing 1000 ng internal standard (B), a quality control plasma sample of antiretroviral drugs (C) and a sample from a patient who was given raltegravir 400 mg BID in combination with other antiretrovirals and containing 666 ng/mL of the assayed drug analyzed in presence (D) and in absence of internal standard (E).

Table 1
Intra-day accuracy and precision of plasma quality control samples ($n = 6$)

	10 ng/mL (Low QC)	50 ng/mL (Medium QC)	750 ng/mL (High QC)
Mean observed	9.8	49.0	743.1
Standard deviation	0.4	1.9	9.0
Accuracy (%)[a]	98.3	98.0	99.1
Precision (%)[b]	4.5	3.8	1.2

[a] Expressed as (mean observed concentration/added concentration) × 100.
[b] Correspond to the coefficient of variation.

plasma (Fig. 2A) did not show the presence of any interfering peak at the retention times of interest. Fig. 2B show chromatogram of a 5 ng/mL spiked drug-free human plasma containing 1000 ng internal standard. Analysis of a French Asqualab quality control for antiretroviral drugs is presented in Fig. 2C. The analysis of a patient sample conducted with and without internal standard are shown in Fig. 2D and E, respectively. This patient was treated with raltegravir 400 mg BID plus the boosted protease inhibitor fosamprenavir and the non-nucleoside reverse phase inhibitor etravirine (TMC125). The raltegravi concentration measured 14 h after the last drug intake was 666 ng/mL.

4.3. Calibration curves

Least squares weighted (weighting factor = $1/y$ where y = peak-height ratio) linear regressions were used to calculate the equations relating the peak-height ratio between raltegravir and internal standard and the concentration of the drug. Six seven-points raltegravir calibration curves in human plasma ranging from 5 to 1000 ng/mL were analyzed. The weighted peak-height ratio values of the calibration standards were proportional to the concentration over the range tested. Mean ± of standard deviation of the slopes of these plasma standard curves was slight: $1.289\ 10^{-03} \pm 0.047 \times 10^{-03}$ (CV = 3.7%) with an intercept of 0.3 ± 0.1 ng/mL. The average coefficient of correlation (r^2) was 0.9999 ± 0.0001.

4.4. Intra-day variability of plasma quality control samples

Intra-day variability of the method was studied in assaying six quality control samples at 10 ng/mL (low QC), 50 ng/mL (medium QC) and 750 ng/mL (high QC) the same day. The mean accuracy was 98.3–99.1% of the nominal concentrations with a C.V. of 4.4% or less (Table 1).

4.5. Inter-day variability of plasma quality control samples

Inter-day variability of the method was studied in assaying one set of the quality control samples on six separate occasions. The mean accuracy was 98.3–101.0% of the added concentrations with a C.V. of 6.3% or less (Table 2).

Table 2
Inter-day accuracy and precision of plasma quality control samples ($n = 6$)

	10 ng/mL (Low QC)	50 ng/mL (Medium QC)	750 ng/mL (High QC)
Mean observed	10.1	49.2	752.7
Standard deviation	0.6	2.0	15.3
Accuracy (%)[a]	101.0	98.3	100.4
Precision (%)[b]	6.2	4.0	2.0

[a] Expressed as (mean observed concentration/added concentration) × 100.
[b] Correspond to the coefficient of variation.

Accuracy and precision results were not significantly different when the QC samples were prepared from another lot of drug-free human plasma.

4.6. Absolute recovery

The efficiency of the raltegravir extraction procedure was determined by comparing the slopes of six plasma calibration curves to that of the pure working standards injected directly the same day. Mean absolute recoveries were 66.0 ± 1.5% and 72.5 ± 2.1% for raltegravir and internal standard, respectively.

The absolute recovery of raltegravir is lower than in the original assay using a 96-well liquid–liquid extraction [12]: ranging from 82.8 (5 ng/mL) to 95.6% (750 ng/mL).

4.7. Limit of accurate determination

The lower limit of accurate determination (LLOQ) was arbitrarily set at 2.5 ng/mL i.e. half concentration of the first calibration standard. At this plasma level the mean accuracy of five intra-day determinations was 86% with a precision of 8.1%.

This LLOQ is higher than observed previously (0.2 ng/mL) using a HPLC–MS/MS method [12] but is 9 times lower than the 95% inhibitory concentration of the drug (22 ng/mL). In subjects who received raltegravir 400 mg twice daily alone a geometric mean drug trough plasma concentration of 63 ng/mL was observed [11], 25-fold higher than the LLOQ of the assay.

4.8. Stability of processed samples

Plasma extracts from seven different patients treated with 400 mg BID raltegravir were pooled and the raltegravir plasma concentration calculated from the initial calibration curve was 210 ng/mL. This pooled plasma sample was then re-injected after 1–4 days storage at ambient temperature and at 4 °C in the autosampler and concentrations calculated from the initial standard curve. No difference was observed after 4 days of storage between 4 °C and ambient temperature. Accuracy varied from 96.4 to 104.5%. Therefore, raltegravir was considered to be stable in the tested conditions.

4.9. Analytical interferences

Four sets of quality controls from the French Asqualab external quality control program for antiretroviral drugs were analyzed using the described procedure. In addition patients who were given the recent protease inhibitor darunavir and the new HIV drug candidate TMC125 were also assayed. Among the tested drugs: amprenavir, atazanavir, darunavir, efavirenz, lopinavir, nelfinavir and its M8 metabolite, nevirapine, ritonavir, saquinavir, tipranavir and TMC125, none was found to interfere with internal standard or raltegravir. In addition the polar nucleoside reverse transcriptase inhibitors: abacavir, didanosine, emtricitabine, lamivudine, tenofovir and zidovudine were not retained on the column.

In addition, raltegravir predose plasma samples analyzed with and without internal standard showed the absence of raltegravir metabolites at the retention time of the internal standard.

4.10. Clinical application of the assay

Plasma samples from 21 patients receiving 400 mg BID of raltegravir in association with other antiretrovirals were assayed. Trough drug concentrations obtained 9.5–15.5 h after the last intake ranged from 15 to 1197 ng/mL. In patients receiving raltegravir 400 mg twice daily alone, a 142 nM (63 ng/mL) geometric

mean trough plasma concentration (C12h) was observed. Raltegravir pharmacokinetics varied significantly with an inter-subject coefficient of variation of 212% for its C12h (11). The predose concentrations we found confirm this marked inter-patient variability.

5. Conclusion

This liquid chromatographic assay with fluorescence detection allows the quantitative determination of raltegravir plasma concentrations at levels observed in clinical settings without need of an expensive MS/MS detector and may be used to perform therapeutic drug of this HIV integrase inhibitor even if the run-time of the assay is significantly longer.

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