Journal of Chromatography B, 867 (2008) 165–171

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A sensitive HPLC–MS–MS method for the determination of raltegravir in human plasma

Mary C. Long, Chantelle Bennetto-Hood, Edward P. Acosta *

Division of Clinical Pharmacology, University of Alabama at Birmingham School of Medicine, United States

ARTICLE INFO

Article history:
Received 12 November 2007
Accepted 26 March 2008
Available online 1 April 2008

Keywords:
HIV
Integrase inhibitor
Mass spectrometry
Human
Raltegravir

ABSTRACT

This work describes an assay system that has been developed to quantify raltegravir concentrations in human plasma using a liquid–liquid extraction technique paired with HPLC separation and MS–MS detection. The dynamic range of this assay extends from 1 to 3000 ng/mL, with a coefficient of determination (r^2, mean ± SD) of 0.9992 ± 0.0002. The mean precision values for calibration standards ranged from 0.6% to 3.0%, while accuracy values were 96.5–104.3%. This procedure is an accurate, precise, and sensitive method for raltegravir quantitation and was successfully validated using external proficiency testing.

1. Introduction

Raltegravir (RAL, MK-0518, Isentress, Fig. 1) is the first drug in the new class of integrase inhibitors to be approved by the U.S. Food and Drug Administration for treatment-experienced patients with HIV-1 infection [1]. The mechanism of action for RAL involves blocking the insertion of viral DNA into the cellular genome by binding to the acceptor DNA-binding site of the integrase–viral DNA complex [2]. Once integration is blocked, HIV-1 can no longer replicate, and the viral life cycle is interrupted. Since this drug has a novel mechanism of action, RAL retains activity against strains of HIV-1 that are resistant to other classes of drugs, including protease inhibitors, nucleoside and non-nucleoside reverse transcriptase inhibitors. Integrase is the only HIV-1 enzyme for which there was no inhibitor prior to this time [3,4] and represents a new alternative for patients who are failing antiretroviral treatment due to resistance mutations.

In clinical studies, RAL has proven effective at rapidly decreasing viral burden in patients receiving 400 mg b.i.d., with a concomitant increase in $CD4^+$ cell count in patients with advanced HIV-1 infection who had previously failed therapy with triple class-resistant virus, and limited treatment options [5]. Furthermore, in treatment-naïve patients receiving RAL at 100, 200, 400, or 600 mg twice daily, there was a 2-fold log reduction in HIV-1 plasma viral load over 10 days that was similar to the change that is seen with a combination of three active drugs [6]. The IC_{95} for RAL is 33 nM (15 ng/mL) in the presence of 50% human serum, and typical trough concentrations for 400 mg b.i.d. were 200 nM (89 ng/mL) [3]. Since trough concentrations exceeded the IC_{95} by a large margin, there is a decreased likelihood of developing drug resistance in patients who adhere to their drug regimen [3]. When used as monotherapy, or in combination with other antiretroviral drugs, RAL has demonstrated sustained antiviral activity with little toxicity over 16–24 weeks of treatment [5,6]. The side effects reported were generally mild and included diarrhea, nausea, vomiting, fatigue, and headache, but rates were comparable to placebo [5,6]. One report of elevated blood bilirubin was reported, but was mild and resolved with time even though treatment was continued [5].

Studies of the metabolism and disposition of RAL indicate that the major mechanism of clearance in humans is via glucuronidation by UDP-glucuronosyltransferase 1A1 (UGT1A1), with urinary and fecal elimination [4]. Co-administration of RAL with atazanavir, a potent inhibitor of UGT1A1, demonstrated a 1.7-fold increase in the AUC of RAL, whereas co-administration with rifampin, a UGT1A1 inducer, resulted in a 40% decrease in the AUC for RAL [4]. When atazanavir and RAL were co-administered, the antiretroviral effect and response rate was greater than for other antiretroviral combinations, however, it is difficult to tell if this effect would be sustained with a larger sample size [5]. Since RAL will be administered as part of a multi-drug regimen, the lack of significant interactions with other antiretroviral agents is clinically advantageous.

* Corresponding author at: University of Alabama at Birmingham School of Medicine, Division of Clinical Pharmacology, 1530 3rd Avenue South, VH 116, Birmingham, AL 35294-0019, United States. Tel.: +1 205 934 2655; fax: +1 205 934 6201.
E-mail address: eacosta@uab.edu (E.P. Acosta).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.03.022

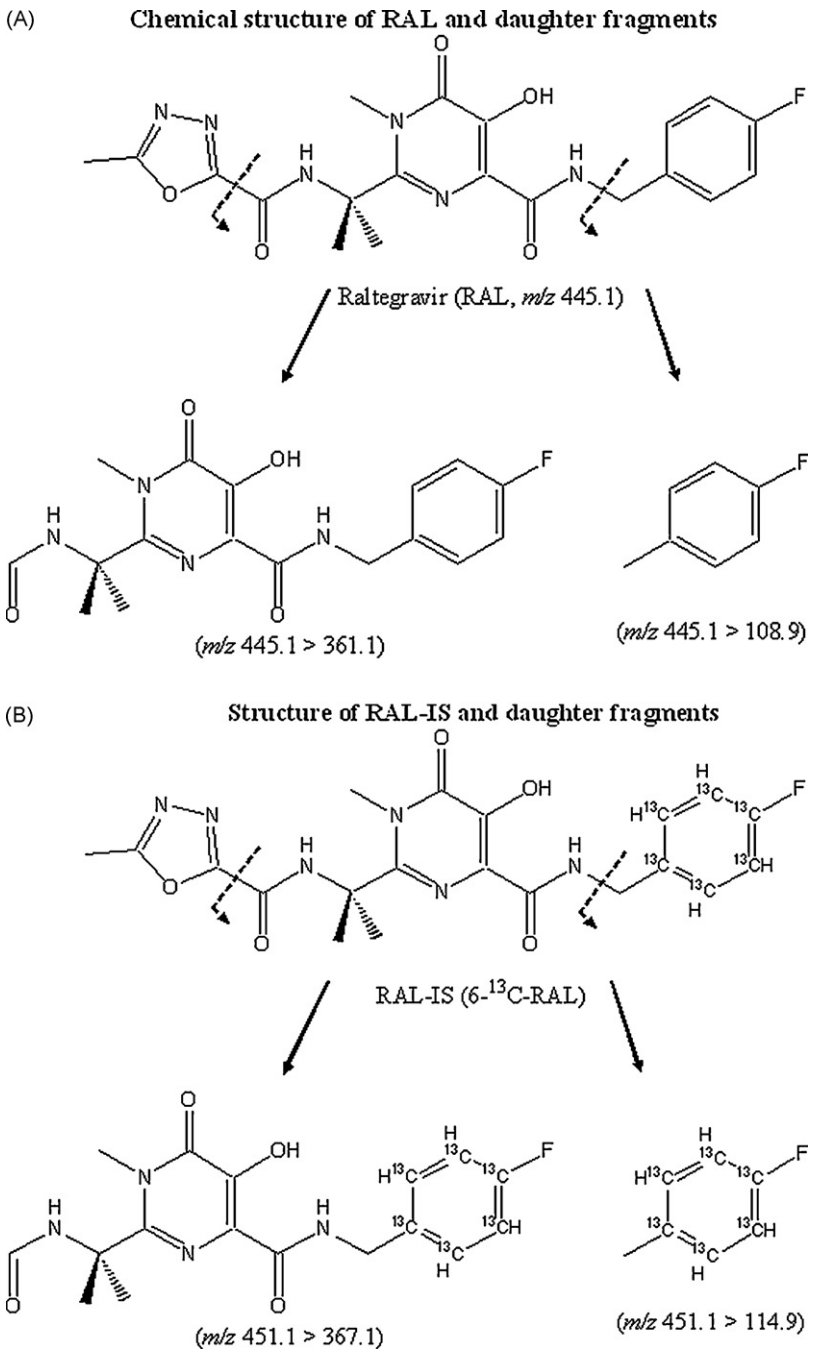

Fig. 1. The chemical structures of (A) RAL and (B) RAL-IS, and their respective daughter fragments.

RAL is the first integrase inhibitor to be approved, has an excellent safety and tolerability profile, and has demonstrated efficacy, therefore, it has the potential to alter current treatment paradigms. It is currently approved for treatment-experienced patients, but holds promise to become a first-line antiretroviral agent. Therefore, we have developed an assay for determining the concentration of RAL in human plasma using a liquid–liquid extraction and high-pressure liquid chromatography (HPLC) separation coupled with triple-quadrupole mass spectrometry (MS–MS) detection. This assay is adapted from a previously published method that was used to support 18 clinical studies during Phases I through III of clinical development [7]. The major advantage of this protocol over the previously published method is the improved dynamic range of the assay, which is set to encompass mean peak and trough concentrations that have been measured in previous studies (mean troughs as low as 23 ng/mL, and mean peaks up to 2.9 μg/mL) [3,6].

2. Experimental

2.1. Reagents

All solvents, including HPLC-grade methanol, isopropanol, methylene chloride, acetonitrile (ACN) and hexane were obtained from Fisher Scientific (Pittsburgh, PA). Ammonium acetate and formic acid (88%) were obtained from Fisher Scientific (Pittsburgh, PA). Merck Research Laboratories (West Point, PA) provided both

RAL potassium salt and a stably labeled isotope of RAL ($^{13}C_6$-RAL), which served as the internal standard (RAL-IS). Ethylenediamine tetraacetic acid (EDTA) treated plasma was obtained from Biological Specialties Corporation (Colmar, PA). Water was purified on-site using a Barnstead Mega-Pure system (Dubuque, IA).

2.2. Instrumentation and software

A Waters 2695 HPLC coupled with a MicroMass Quattro Micro mass spectrometer (Waters Corp., Milford, MA) were utilized for the separation and detection of RAL. Both the HPLC and mass spectrometer were controlled remotely using MassLynx software v 5.0 (Waters Corp., Milford, MA.). Data analysis was performed with the QuanLynx module that accompanies MassLynx.

2.3. Calibration standard and quality control preparation

A 1 mg/mL master stock solution of RAL was prepared in 1:1-ACN:water. This solution was diluted to make a series of standard curve working solutions at 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 3, 6, 10, 15, 20, 25, and 30 μg/mL. A 200 μg/mL stock solution of RAL-IS was prepared in 1:1-ACN:water and diluted to 1 μg/mL. For preparation of calibration curves, 20 μL of the appropriate level of calibration standard and 20 μL of RAL-IS was added to 200 μL of freshly thawed EDTA plasma in a 16 mm × 125 mm borosilicate glass culture tube, and mixed. A separate weighing was used in order to prepare a 1 mg/mL quality control master stock solution in 1:1-ACN:water. The quality control master stock was diluted to 0.03, 5.4, and 27 μg/mL for low, mid, and high-level quality control working solutions. Plasma quality controls were prepared by addition of 2 mL of the appropriate level of quality control working solution to 18 mL of EDTA plasma, for final concentrations of 3, 540, and 2700 ng/mL. Aliquots of 800 μL were prepared and stored at −80 °C prior to use. All master stocks, working standards, and quality control standards were stored at room temperature.

2.4. Sample preparation

Calibration standards were prepared as described above. Additional samples consisted of 200 μL of unknown (patient) or quality control plasma. RAL-IS and 1:1-ACN:water (20 μL each) were added to all unknown and quality control samples. Blank (plasma only with 40 mL of 1:1-ACN:water, no internal standard) and 'zero' (plasma with internal standard and 20 μL of 1:1-ACN:water) samples were included in every run. An adaptation of a previously published liquid–liquid extraction was employed in order to separate RAL from plasma components [7]. Briefly, all calibration standards, quality control samples, and unknown samples were acidified by the addition of an equal volume of 200 mM ammonium acetate, pH 4, and then briefly mixed by vortexing in a 16 × 125 borosilicate glass culture tubes. RAL was extracted by the addition of 1.5 mL of 1:1-methylene chloride:hexane, and samples were thoroughly mixed by vortexing for 30 s prior to centrifugation (2750 × *g*) for 5 min. Following centrifugation, the aqueous component was frozen in a dry ice–isopropanol bath and the organic component was decanted into a separate 2.0 mL microcentrifuge tubes. Organic phase was evaporated under a stream of nitrogen in a 40 °C water bath using a TurboVap LV concentration workstation (Caliper Life Sciences, Hopkinton, MA). Dried samples were reconstituted to 200 μL in HPLC mobile phase (47.5–0.1% formic acid, 52.5% methanol), and mixed by vortexing for 30 s. Samples were then transferred to low-volume inserts in HPLC vials and loaded into the autosampler. Extraction efficiency was tested by comparing the recovery of extracted quality control standards (9, 540, and 2700 ng/mL, as described above) to post-extraction spike solutions and test spike solutions prepared in 1:1-ACN: water.

Table 1
Settings for MS–MS detection of raltegravir

Source (ES^+)	Settings	Analyzer	Settings
Capillary (kV)	3.50	Low Mass 1 Resolution	15.0
Cone (V)	30.00	High Mass 1 Resolution	15.0
Extractor (V)	2.00	Ion energy 1	0.3
RF Lens (V)	0.0	Entrance	0
Source temperature (°C)	107	Collision	20
Desolvation temperature (°C)	450	Exit	1
Cone gas flow (L/h)	50	Low Mass 2 Resolution	15.0
Desolvation gas flow (L/h)	800	High Mass 2 Resolution	15.0
		Ion energy 2	2.0
		Multiplier (V)	650
		Gas cell pirani pressure (mbar)	$3.2e^{-3}$

2.5. HPLC separation and MS–MS detection

All samples were subjected to separation using a Waters 2695 HPLC with an ACE C_{18} column (3 μM, 50 mm × 3 mm, MacMod analytical, Chadds Ford, PA). Separation was achieved with an isocratic run, using the mobile phase detailed above. Injections consisted of 20 μL of each sample, the flow rate was set at 0.2 mL/min, and the overall run time was 7 min. The injection port was washed in between runs with 10 loop volumes of 50% methanol. RAL and RAL-IS were detected as they eluted from the column using MS–MS detection in the electrospray positive (ES^+) mode. RAL and RAL-IS eluted at 3.8 min and were detected by monitoring the following transitions for protonated daughters $[M+H]^+$ of RAL: *m/z* RAL, 445.1 → 109.0 and 445.1 → 361.1; *m/z* RAL-IS 451.1 → 115.0 and 451.1 → 367.1. The relative abundance of these daughters were 39% (*m/z* 445.1 → 361.1) and 61% (*m/z* 445.1 → 109.0). Settings for the mass spectrometer are listed in Table 1. Traces correlating to the transitions *m/z* 445.1 → 109.0 for RAL and *m/z* 451.1 → 367.1 for RAL-IS were integrated and concentration values were quantitated relative to internal standard area. Calibration curves were created using a 1/concentration^2 weighted linear regression.

2.6. Assay validation

Validation runs containing the full calibration curve, blank samples, 'zero' samples, six replicates each of the lower limit of quantitation (LLOQ, 1 ng/mL), low, mid, and high-level quality control samples were run on six different days. Four different lots of EDTA-treated human plasma were used during the validation process. The results of these runs determine the inter-day and intra-day precision and accuracy values. Acceptance criteria were such that calibration curves had to have an r^2 of ≥0.98, and the back-calculated values of standards used to create calibration curves were required to be within ±15% of the nominal concentration. Standards could be excluded if they did not meet these criteria, but sequential standards could not be excluded. Concentrations of LLOQ and quality control samples were determined from calibration curves created with each run and at least four out of six of these had to be within 15% of nominal value.

RAL assay specificity was tested by extracting RAL in the presence of currently approved antiretroviral drugs at concentrations near the maximum plasma concentration that could be expected for each drug. The antiviral drugs tested include: abacavir, acyclovir, amprenavir, atazanavir, darunavir, didanosine, efavirenz, emtricitabine, ganciclovir, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, tenofovir, zalcitabine, and zidovudine. Assays were extracted in triplicate and run sequen-

tially to verify that the secondary drug would not carryover and interfere with subsequent runs.

The ability to accurately dilute a sample that has concentrations above the upper limit of quantitation was examined by spiking a plasma sample with RAL at a level of 10,000 ng/mL (the upper limit of quantitation is 3000 ng/mL). Triplicate sets of three dilutions (1:5, 1:10 and 1:20) were prepared in human plasma in order to assess the ability to dilute samples that are above the upper limit of quantitation.

In order to verify that the performance of the present assay was comparable to the previously reported assay, we cross-validated our assay with both quality control and external proficiency samples provided by Merck Research Laboratories (MRL, West Point, PA). The proficiency studies were blinded in terms of the unknown samples and our results had to be within 20% of the value determined by MRL in order to pass. Duplicate assays were run with replicate samples.

The ability to re-inject samples following a weekend in the autosampler was investigated by extracting and running a full validation assay on a Friday afternoon. The unused volume remained in the autosampler over the weekend and all samples were re-analyzed on the following Monday in order to simulate equipment failure. The resulting calibration curves and sample values were calculated within each run (runs 1 and 2) and cross-checked between runs (the curve from run 1 was used to calculate sample values from run 2). This experimental design permitted us to verify the integrity of results obtained after samples sat over a weekend for both a full run and re-analysis of partially obtained data if equipment failed and sample volumes were inadequate for re-injection (e.g., some data were obtained on a

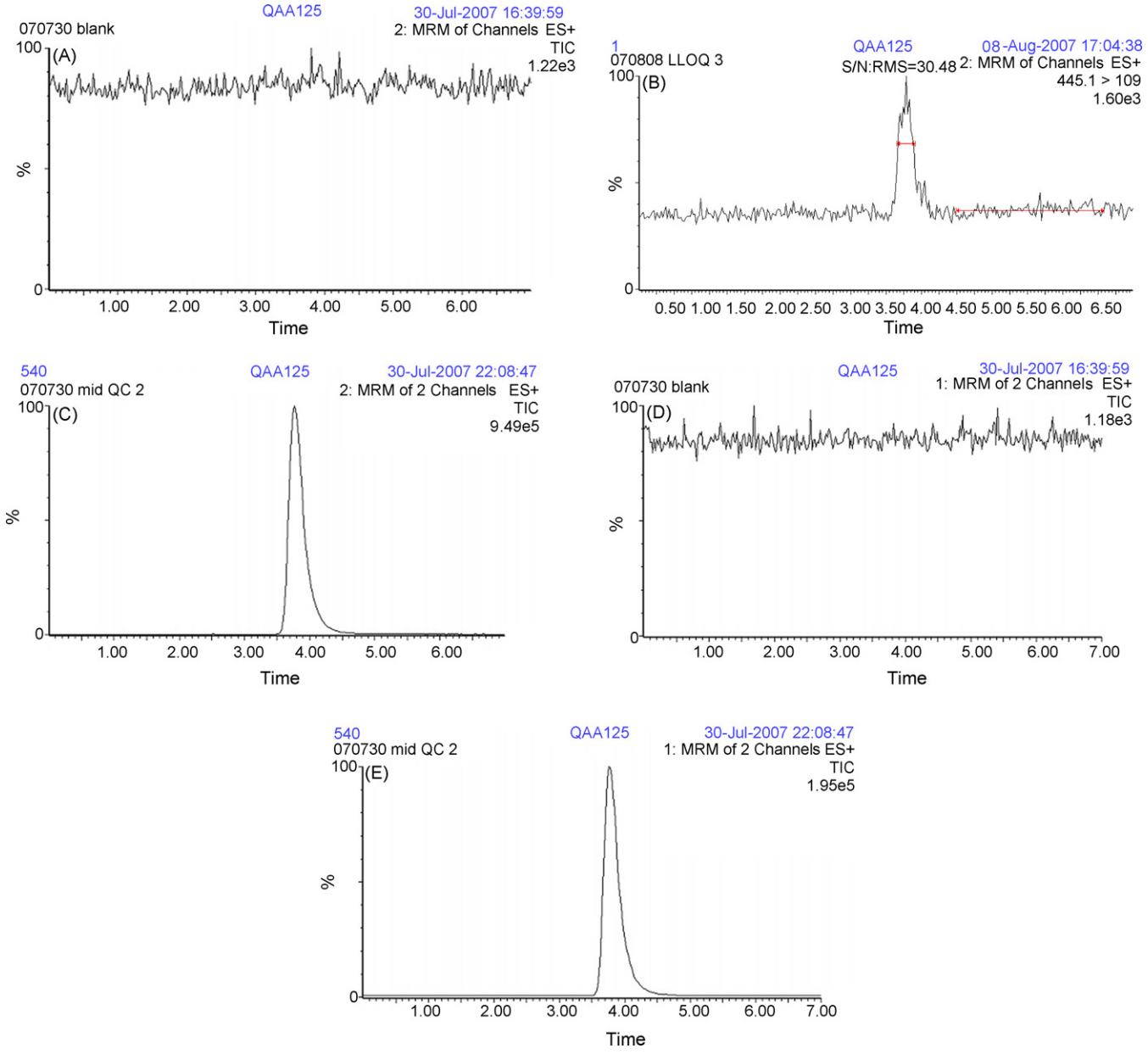

Fig. 2. Representative chromatograms obtained by applying the present method to: (A) a blank extracted EDTA-plasma sample monitored on RAL channels, m/z 445.1 > 108.9 and m/z 445.1 > 361.1; (B) an example of the lower limit of quantitation (1 ng/mL) sample demonstrating the signal to noise ratio for this calibrator using the single channel m/z 445.1 > 108.9; (C) a mid-range quality control sample (540 ng/mL); (D) a blank, extracted EDTA-plasma sample monitored in RAL-IS channels, m/z 451.1 > 114.9 and m/z 451.1 > 367; and (E) internal standard (100 ng/mL) extracted concomitantly with the RAL mid-range quality control standard.

Table 2
Recovery of RAL

	Spiked mobile phase (mean peak area)	Post-extraction spike (mean peak area)	Extracted (mean peak area)	%Recovery[a]	Mean matrix effect (%)[b]
Low (9 ng/mL)	1078	1294	1216	94	120
Mid (540 ng/mL)	63,990	74,280	66,430	89	116
High (2700 ng/mL)	335,900	383,100	335,439	88	114

[a] %Recovery is calculated as (mean extracted peak area/post-extraction spike peak area) × 100%.
[b] Mean matrix effect is calculated as (mean post-extraction spike peak area/mean spiked mobile phase peak area) × 100%.

Friday, and the remaining data acquired on the following Monday).

3. Results

3.1. Chromatography, detection and quantitation of RAL

Initial optimization of the triple-quadrupole mass spectrometer for the detection of an analyte begins with optimizing for the parent compound, in this case RAL, in the single-quadrupole mode. This step of optimization revealed large peaks at $[M+H]^+$ for RAL at m/z 445.1, 467.1, and 483.1. The peak at m/z 445.1 correlated with the expected molecular mass of RAL, whereas the peaks at m/z 483.1 and 467.1 were due to the formation of potassium and sodium adducts of RAL, respectively. The predominant peak contained about 80% of the signal and correlated with the formation of sodium adducts. Conditions were optimized for detection of RAL and its daughter ions (Table 1) and plasma samples spiked with RAL were extracted. Acidification of the plasma samples with 200 mM ammonium acetate, pH 4, during extraction forced the stoichiometry of the system such that RAL became the major peak, with ~74% of the total signal for the drug, while sodium and potassium adducts accounted for 14.5% and 11.4% respectively. Experiments were performed with a full calibration curve, and the ratios held across all concentrations, therefore adduct formation was proportional to the concentration of RAL in solution and we could detect daughters of RAL m/z of 108.9 and 361.1 to monitor the elution of RAL from the column. Two daughters were monitored in order to assure that the peak of interest was RAL and not an interfering peak. However, only a single channel for each RAL and RAL-IS (m/z 445.1 → 108.9) and 451.1 → 367.1) was used for quantitation (Fig. 2).

Under the conditions set forth, the separation of RAL and RAL-IS from human plasma components was successfully achieved (Fig. 2). No interfering peaks were detected across four different lots of human plasma utilized during assay validation, as seen in extracted blank samples. The lower limit of quantitation for RAL was set at 1 ng/mL, with a mean signal:noise (S/N) ratio of 36.2 (a S/N ratio of ≥5 is considered acceptable for the LLOQ), and a detection limit of 0.6 ng/mL. RAL maintained its linearity from 1 to 3000 ng/mL without any loss of signal intensity at the upper end of the curve. The coefficient of determination for the six validation runs was 0.9992 ± 0.0002 (r^2, mean ± SD). Carryover from the injection port became problematic at concentrations higher than 3000 ng/mL, even with washing in-between samples with 50% methanol. At concentrations of RAL ≥6000 ng/mL, carryover was equivalent to ~45 area units, approximately 10% of the area of the LLOQ peak (1 ng/mL).

3.2. Matrix effect, interferences and recovery

The absolute matrix effect was determined by comparing the peak areas obtained from mobile phase spiked with low, mid, and high concentrations of RAL (9, 540, and 2700 ng/mL, respectively), post-extraction spiked samples, and extracted spike samples [8]. The matrix effect was determined by comparing the mean areas of post-extraction spike samples to clean spike peak areas. A positive matrix effect of 117 ± 3% was measured at all concentrations. Recovery was calculated by comparing the peak areas of mean spiked mobile phase areas to the mean area of extracted controls (Table 2). The mean recovery value was determined to be 90.3 ± 3.2% (Table 2). Since patients taking RAL will be administered other antiretroviral drugs concomitantly, this assay was performed in the presence of commonly used antiviral drugs mentioned previously (see Section 2.6). Assays were run in triplicate, and no drugs were found to interfere with the performance of this assay system (data not shown).

3.3. Assay validation

Each validation run contained blank and 'zero' control samples, a full 14-point curve, plus six replicates each of LLOQ, low, mid and high quality control standards (1, 3, 540 and 2700 ng/mL, respectively). Inter-day precision values calculated from the back-calculated concentrations for each of the 14 curve standards ranged from 0.6% to 2.7%, while accuracy ranged from 96.5% to 104.2% (Table 3). Concentrations determined for the LLOQ, low, mid and high quality control standards were utilized to calculate the intra-day precision and accuracy values (Table 4). The mean intra-day precision values for the six validation assays ranged from 4.8% to 6.8%, while the mean accuracy values were 102% to 109% (Table 4). The quality of the standard curves was evaluated by the mean coefficient of determination ($r^2 = 0.9992 \pm 0.0002$) and the reproducibility of the slope and intercept; mean slope was 0.88 ± 0.03 and intercept values ranged from −0.14 to 0.04 (mean ± standard error = -0.06 ± 0.07).

The ability to use partial volumes or dilution of samples that are above the upper limit of quantitation was determined by test-

Table 3
Inter-day precision and accuracy for RAL in four different plasma lots

Standard concentration (ng/mL)	Mean assayed concentration (ng/mL, $n = 6$)	Accuracy[a] (%)	Precision[b] (%)
1	1.0	100.0	1.2
3	3.05	101.7	2.2
6	5.79	96.5	2.3
10	9.74	97.4	3.0
30	29.3	97.7	2.7
60	59.6	99.3	1.7
100	98.0	98.0	1.6
300	295.5	98.5	1.5
600	625.5	104.2	1.2
1000	1005.7	100.6	0.9
1500	1530.8	102.1	0.6
2000	2021.7	101.1	0.7
2500	2531.7	101.3	0.6
3000	3028.3	100.9	0.7

[a] Accuracy was determined as (mean assayed concentration/nominal standard concentration) × 100%.
[b] Precision values are the %CV for these six determinations.

Table 4
Representative intra-day variability for RAL

Nominal concentration (ng/mL)	Measured concentration (ng/mL, mean ± SD)	Precision[a] (%)	Accuracy[b] (%)
A. Inter-day (n = 36)			
1.0	1.04 ± 0.07	6.8	104
3.0	3.27 ± 0.16	4.8	109
540.0	548.3 ± 27.8	5.1	102
2700.0	2846 ± 154	5.4	105
B. Intra-day 1 (n = 6)			
1.0	1.02 ± 0.07	6.9	102
3.0	3.28 ± 0.13	3.9	109
540.0	551.8 ± 12.1	2.2	102
2700.0	2829 ± 131	4.6	105
Intra-day 2 (n = 6)			
1.0	1.11 ± 0.08	7.5	111
3.0	3.38 ± 0.08	2.5	113
540.0	544.7 ± 10.2	1.9	101
2700.0	2855 ± 45.6	1.6	106
Intra-day 3 (n = 6)			
1.0	1.00 ± 0.03	3.4	100
3.0	3.21 ± 0.09	2.7	107
540.0	550.7 ± 7.0	1.3	102
2700.0	2875 ± 56.7	2.0	106
Intra-day 4 (n = 6)			
1.0	1.09 ± 0.04	3.7	109
3.0	3.33 ± 0.10	3.0	111
540.0	581.6 ± 16.9	2.9	108
2700.0	3011 ± 140	4.7	111
Intra-day 5 (n = 6)			
1.0	1.03 ± 0.06	5.8	103
3.0	3.34 ± 0.17	5.0	111
540.0	561.4 ± 14.8	2.6	104
2700.0	2904 ± 91.6	3.2	108
Intra-day 6 (n = 6)			
1.0	0.98 ± 0.04	3.7	98
3.0	3.06 ± 0.13	4.1	102
540.0	500 ± 14.6	2.9	93
2700.0	2602 ± 78.1	3.0	96

[a] Precision values are the %CV for these six determinations.
[b] Accuracy was determined as (mean assayed concentration/nominal standard concentration) × 100%.

ing three dilutions of a 10,000 ng/mL sample. Mean accuracy values were 100.5%, 96.3%, and 101.7% for the 1:5, 1:10 and 1:20 dilutions, respectively, and precision values ranged from 0.3% to 3.1% across dilutions (Table 5). These results indicate that dilutions up to 1:20 are acceptable for quantitation of samples and should cover the range of systemic peak concentrations measured in patient samples.

External proficiency samples were tested during validation of the current method. Concentration values determined for the low, mid and high quality control samples provided by MRL ranged from −10.8% to 3.3% of nominal, and unknown samples intended to simulate a patient's concentration–time profile ranged from 0.7% to 11.5% of the nominal concentration (data not shown). A total of 12 concentration–time points were used for this cross-validation; the correlation coefficient (r) between our results and those of MRL was

Table 5
Precision and accuracy of partial volume extractions

	Dilution 1:5	Dilution 1:10	Dilution 1:20
Nominal concentration (ng/mL)	10,000	10,000	10,000
Mean (n = 3, ng/mL)	10,053	9,626	10,171
Standard deviation	73.3	30.7	315.1
Precision (%)	0.7	0.3	3.1
Accuracy (%)	100.5	96.3	101.7

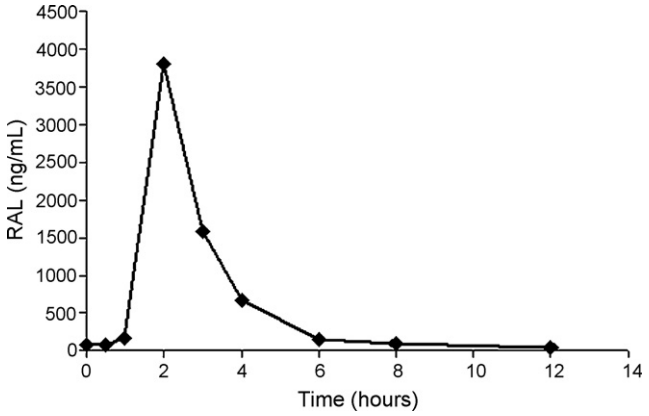

Fig. 3. A representative steady-state concentration–time profile for a single patient taking RAL.

0.99. The current assay was considered equivalent to the existing assay based on the results of this cross-validation.

The quality of the calibration curves generated after re-injection of samples 3 days apart resulted in similar slopes (0.12% different), intercepts and correlation coefficients (0.01% different), indicating that the curves generated from re-injection of samples after sitting for 3 days in the autosampler results in nearly identical calibration curves. The difference between the determined mean values for LLOQ, low, mid, and high-level quality control samples when analyzed independently after 3 days in the autosampler ranged from 0% to 4.8% (Table 6). Therefore, if enough sample volume exists to re-analyze an entire analytic run, the results are equivalent between same day analysis and analysis after 3 days at room temperature. When there is inadequate sample volume to permit a full re-analysis, our results indicate that the results acquired several days apart can be combined. These conditions were simulated by analyzing LLOQ, low, mid, and high-level quality control samples acquired on day 3 (Monday) off of a calibration curve generated on day 0 (Friday). The difference between re-analyzed samples (day 3) and values obtained on day 0 ranged from 0.2% to 4.9%, indicating that these results are equivalent (Table 6).

3.4. Application of the analytical method

This method was applied to determine RAL concentrations in a single HIV-infected patient. Steady-state, intensive pharmacokinetic samples were taken at 0, 0.5, 1, 2, 4, 6, 8, and 12 h post-dose. The resulting concentration–time profile is shown in Fig. 3.

4. Discussion

RAL is a first-in-class HIV integrase inhibitor to become available for use in treatment-experienced patients. Due to its unique mechanism of action, rapid and potent antiviral effect, and excellent safety and tolerability profile, this new drug will likely become extensively used and considerable research remains to be completed. An HPLC–MS–MS assay has already been reported for this drug [7]. Although the previously reported assay was well developed, we found that assay to be specific to the type of equipment utilized in its development, and not readily adaptable to other HPLC and mass spectrometer systems. The current work describes an adaptation of that assay that makes it more universally applicable and extends the dynamic range of the assay 6-fold in order to encompass the range of peak and trough concentrations that are found patients receiving the standard dose of 400 mg twice daily. Stability experiments have not been reported here as our results are similar to those previously published, with excellent short-term and long-

Table 6
Re-injection stability following a weekend at room temperature

	Run 1	Run 2	Re-analysis[a]	Between-run %difference[b]	Re-analysis %difference[c]
Slope	0.9426	0.9415		0.12	
Intercept	−0.07388	−0.04988			
r^2	0.9988	0.9987		0.01	
LLOQ	1.04 ± 0.08	0.991 ± 0.06	1.01 ± 0.06	4.8	2.9
Low QC	2.77 ± 0.19	2.89 ± 0.12	2.91 ± 0.12	4.2	4.9
Mid QC	469 ± 23	469 ± 21	468 ± 21	0	0.2
High QC	2392 ± 23	2390 ± 14	2387 ± 14	0.08	0.2

[a] Values determined using the calibration curve from run 1 and the raw data from run 2.
[b] Between-run %difference was determined by the following equation: |(run 1 − run 2)|/((run 1 + run 2)/2).
[c] Re-analysis %difference was determined by the following equation: |(run 1 − re-analysis value)|/((run 1 + re-analysis value)/2)

term stability as well as thermo stability toward both low and high temperatures [7].

Extraction of RAL and HPLC separation were similar between our assay systems, with a few noteworthy exceptions. The previously described liquid–liquid extraction was performed using a Tomtek Quadra 96, and analyte recovery ranged from 83% to 96% [7]. We adapted the assay for extraction using test tubes to make it more universally applicable, and our recovery ranged from 99.9% to 112.8%, which is comparable to the previous results. HPLC conditions were similar with one very important exception. Ethylenediamine tetraacetic acid was excluded from our HPLC buffers because it precipitated in our instrument, causing problems with fluctuating backpressure and overpressure warnings to occur repeatedly. EDTA was presumptively included in the previous assay system in order to minimize adduct formation with sodium and potassium (described above in Section 3). These adducts can be seen during direct infusion of RAL, however acidifying plasma samples to pH 4 prior to extraction shifts the relative abundance of adducts such that RAL becomes the abundant species (∼75% of the total signal) and is sufficient to warrant the removal of EDTA from the HPLC buffers.

Another major difference between these two assay systems is the switch away from atmospheric pressure chemical ionization ($APCI^+$) to ES^+ for MS–MS detection. Both assay systems monitored the formation of similar daughters, but the present assay monitored the formation and ratios of two different daughter species in order to verify that the peak of interest was indeed RAL. The signal generated in the ES^+ mode was an order of magnitude larger than the signal generated in the $APCI^+$ mode on our system, permitting us to decrease the LLOQ due to greatly enhanced signal intensity. The positive matrix effect that we measured using this technique does not affect the performance of this assay method, but limits its application to the quantitation of only samples that have been similarly prepared from matching matrices. Although the $APCI^+$ mode minimizes adduct formation, we had greater sensitivity and a greatly improved dynamic range in the ES^+ mode. While our current assay ranges from 1 to 3000 ng/mL, this drug shows no sign of ion suppression at high concentrations. Indeed, the limiting factor for the upper end of our assay was not signal strength, but rather the ability of our autosampler to handle such high concentrations without analyte carryover. There is considerable flexibility in this assay system for adaptation to different needs. For example, the lower end of the curve can be reduced by addition of a concentrating step, or lower sample volume needs can be met without compromising the upper or lower ends of the curve because there is no concentration step.

The current assay has been successfully validated and used to measure plasma RAL concentrations in clinical samples. As a result of the increased dynamic range of this assay, fewer samples will have to be diluted and re-analyzed, therefore decreasing turnaround time for patient samples. In short, the assay system reported here represents an improvement over the existing assay because it has been adapted for equipment that is universally available and has a greatly improved dynamic range.

Acknowledgements

Supported by grants U01 AI068636, U01 AI068632, and RO1 AI058696 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health.

References

[1] AIDS Patient Care STDS 21 (2007) 702.
[2] A. Savarino, Retrovirology 4 (2007) 21.
[3] M. Iwamoto, L.A. Wenning, A.S. Petry, M. Laethem, M. De Smet, J.T. Kost, S.A. Merschman, K.M. Strohmaier, S. Ramael, K.C. Lasseter, J.A. Stone, K.M. Gottesdiener, J.A. Wagner, Clin. Pharmacol. Ther. (2007).
[4] K. Kassahun, I. McIntosh, D. Cui, D. Hreniuk, S. Merschman, K. Lasseter, N. Azrolan, M. Iwamoto, J.A. Wagner, L.A. Wenning, Drug. Metab. Dispos. 35 (2007) 1657.
[5] B. Grinsztejn, B.Y. Nguyen, C. Katlama, J.M. Gatell, A. Lazzarin, D. Vittecoq, C.J. Gonzalez, J. Chen, C.M. Harvey, R.D. Isaacs, Lancet 369 (2007) 1261.
[6] M. Markowitz, J.O. Morales-Ramirez, B.Y. Nguyen, C.M. Kovacs, R.T. Steigbigel, D.A. Cooper, R. Liporace, R. Schwartz, R. Isaacs, L.R. Gilde, L. Wenning, J. Zhao, H. Teppler, J. Acquir. Immune Defic. Syndr. 43 (2006) 509.
[7] S.A. Merschman, P.T. Vallano, L.A. Wenning, B.K. Matuszewski, E.J. Woolf, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 857 (2007) 15.
[8] P.J. Taylor, Clin. Biochem. 38 (2005) 328.