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A novel ultrasensitive LC-MS/MS assay for quantification of intracellular raltegravir in human cell extracts

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Abstract

An assay using ultrahigh pressure liquid chromatography and mass spectrometry detection was developed and validated for measurement of the HIV integrase inhibitor raltegravir (MK-0518) in human cell extracts. The assay is designed to utilize 200 μ l of 70% MeOH cell extract derived from human peripheral blood mononuclear cells or human tissue samples. The assay is linear over a range from 0.0023 to 9.2 ng ml⁻¹. The average % CV (SD/Mean)*100 and % Deviation ((observed – target)/target)*100 were less than 20% at the lower limit of quantification and less than 15% over the range of the curve. This assay is an accurate and highly sensitive method for determining raltegravir concentrations in cellular extracts with a lower limit 40 to over 100-fold lower than other methods in the literature. We also present a new processing method where a rapid spin through oil produced a significant increase in apparent intracellular raltegravir concentration compared with conventional processing.

Keywords

Raltegravir; Intracellular; HIV; LC-MS/MS; Integrase Inhibitor

1. Introduction

Raltegravir (RAL, MK-0518, Isentress®) is the first integrase inhibitor approved by the FDA for the treatment of adults and children older than two years with human immunodeficiency virus (HIV)-1 infection [1, 2]. Integrase inhibitors exert their effects by preventing the insertion of viral DNA into the genome of the cell thereby suppressing viral replication [1, 2]. Clinically, raltegravir is well tolerated, and has been shown to have a potent anti-HIV effect. A trial in antiretroviral-naïve adults compared raltegravir with efavirenz, both given in combination with tenofovir disoproxil fumarate and emtricitabine. At week 48 of therapy, 86.1% of the raltegravir group compared with 81.9% of the efavirenz achieved the primary endpoint of < 50 copies ml⁻¹ of HIV-RNA in plasma [3]. At week 156, the rates of viral suppression were 75.4% among those who received raltegravir versus 68.1% for efavirenz [4]. Raltegravir recipients experienced significantly fewer drug-related, adverse events than did efavirenz recipients. Interestingly, the time to suppression of plasma viremia was significantly shorter with raltegravir than efavirenz.

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The pharmacokinetic characteristics of raltegravir are notable for high degrees of intra- and interpatient variability. In HIV-infected persons receiving the recommended dose of 400 mg orally twice daily in the fasting state, the area under the plasma concentration-time curve (AUC) ranged from 1685 to 12770 ng·h ml⁻¹ at steady-state [5]. The highly variable nature of raltegravir's plasma concentrations is inconsistent with the favorable clinical response profile. There may be several mechanisms that complicate the pharmacokinetic/pharmacodynamic or exposure-response profile for this drug. It has been suggested that since raltegravir can be glucuronidated, secondary recycling of raltegravir by the enterohepatic system may occur resulting in a secondary spike in plasma raltegravir concentration and contribute to the variability in plasma concentrations [6]. The residence time of raltegravir on the preintegration complex is longer than the half-life of the free preintegration complex in the cell resulting in functionally irreversible inhibition, which is another contributing factor to the difficulty in finding a predictive pharmacokinetic parameter for efficacy from plasma data. [7,8] Finally, raltegravir exerts its inhibitory action on the integrase enzyme intracellularly; therefore, intracellular concentrations of raltegravir are likely a more accurate metric of virus exposure to the drug and response-related concentrations. To date there have been limited studies of intracellular raltegravir concentrations in peripheral blood mononuclear cells (PBMCs) and quite variable results with ranges of intracellular concentrations from 0% to approximately 10% of corresponding plasma concentrations [6,8]. Factors that may contribute to this variability include cell counting and processing.

This paper describes our development and validation of an ultrasensitive LC-MS/MS based assay to quantify intracellular raltegravir concentrations. Additionally, we compared two different methodologies of cell processing: one typically used for the production of intracellular extracts and an alternate, more rapid procedure involving centrifuging the cells through an oil layer. The raltegravir MS assay was developed to measure minute quantities of raltegravir initially because expected intracellular concentrations were uncertain, and because this assay is intended to support pharmacokinetic studies of intracellular concentrations in PBMCs, the primary target cells for HIV, and in other tissues such as the gut associated lymphoid tissue (GALT), which may serve as a reservoir for the virus. In the case of GALT samples, the number of cells that can be reasonably be obtained by colonoscopy from human volunteers is very low, which increases the needed level of assay sensitivity.

2. Materials and methods

2.1 Chemicals and Cells

Raltegravir (RAL) and C-13 labeled raltegravir (RAL-IS) were obtained from the manufacturer, Merck & Co. Inc. (Rahway, NJ). Acetonitrile (Optima grade), methanol (Optima grade), formic acid (99+% pure), glacial acetic acid (ACS grade) and ammonium acetate (ACS grade) were all obtained from Thermo Fisher Scientific (Fairlawn, NJ). Type I water was produced in the laboratory using a Millipore MilliQ Integral 3 system. Nyosil M25 oil (Nye Lubricants Inc., Fairhaven, MA) was obtained from TAI lubricants Hockessin, DE. Human PBMC's were either isolated from normal donors using elutriation or from patient samples using heparinized cell preparation tubes (Becton Dickinson catalog number 362753, Franklin Lakes, NJ).

2.2 Instruments

The chromatographic system consisted of two Shimadzu Prominence LC-20ADXR pumps, a Prominence SIL20ACXR autosampler, a Prominence CBM-20A controller and a CTO20AC column oven connected to an API 5000 Mass Spectrometer (Applied Biosystem/

Sciex, Foster City, CA) using an ESI interface. The data were acquired and analyzed using Analyst 1.5 software. Pure gases were produced by a Parker Balston SF5500 Gas Generator system (Haverhill, MA). The plate reader was a Biotek Synergy HT (Bio-Tek Instruments, Inc., Winooski, VT). Cells were counted using a Countess cell counter (Invitrogen, Life Technologies Corp., Grand Island, NY).

2.3 Standard curve stock preparation

A 1 mg ml⁻¹ standard stock of raltegravir was prepared in 50% ACN:H₂O from an analytic weighing of raltegravir. This was serially diluted to give a 100 ng ml⁻¹ stock working standard, which was further diluted to make 10X working standards. When corrected for salt content and purity the actual concentrations were of 0.023, 0.046, 0.092, 0.46, 0.92, 4.6, 9.2, 46 and 92 ng ml⁻¹. These were used to make the standard curve by adding 20 µl of these stocks to 180 µl of 70% MeOH. The actual standard curve final concentration ranges from 0.0023 to 9.2 ng ml⁻¹. A later confirmatory study showed good reproducibility over the range from 0.0025 to 10 ng ml⁻¹.

2.4 Quality control and validation sample preparation

A separate weighing of raltegravir solid was used to make a second 1 mg ml⁻¹ stock solution of raltegravir in 50% ACN:H₂O. This solution was diluted to make a 10,000 ng ml⁻¹ preparation stock which was diluted 1:100 to make a final working stock solution of 100 ng ml⁻¹. A 750 µl aliquot of this stock was transferred to a 10 ml class A volumetric flask and diluted to volume with PBMC extract at a concentration corresponding to 2 million cells per ml. When corrected for salt content and purity this produced a 6.9 ng ml⁻¹ QC high (QC-H). The PBMC extract was produced by extracting PBMC with 70% MeOH on ice and removing the cellular debris by centrifugation. The QC-H was diluted with more cell extract to produce concentrations of 0.069 ng ml⁻¹ (QC-medium, QC-M) and 0.0069 ng ml⁻¹ (QC-low, QC-L). Validation samples (VS) were prepared where 80 µl of a 1000 ng ml⁻¹ stock were transferred to a 10 ml volumetric flask and brought to volume with PBMC cell extract. The 8 ng ml⁻¹ validation sample was diluted 1:100 to produce a 0.08 ng ml⁻¹ validation sample. The last two validation samples were produced by diluting volumes of 937.5 and 312.5 µl of the 0.08 ng ml⁻¹ sample to 10 ml. This procedure produced final concentrations (after salt correction) of 7.4, 0.074, 0.0069 and 0.0023 ng ml⁻¹ for the validation samples (VSH, 80% of high std; VSM (medium); and VSL, 3× the LLOQ) and the LLOQ (lower limit of quantitation).

2.5 Internal standard preparation

A working internal standard solution was prepared from reference standard by weighing approximately 1 mg of RAL-IS and dissolving with approximately 1 ml of 50% ACN:H₂O (the exact weight and volume will be the same). This was then diluted to make a 200 ng ml⁻¹ stock solution which was stored at 4 °C. Aliquots were diluted daily with 50% ACN:H₂O to make a working internal standard concentration of 0.5 ng ml⁻¹.

2.6 Sample preparation

Standards were prepared in duplicate by adding 200 µl of water to labeled 1.5 ml snap top centrifuge tubes. This was followed by 180 µl of 70% MeOH, 20 µl of ammonium acetate at pH 4 and 20 µl of internal standard solution (except to the blank). Each of these components was added with an Eppendorf repeater pipette. Finally, 20 µl of standard solution was added to each tube except the blank and blank + IS tubes. The tubes were briefly mixed on a vortex mixer and pulse centrifuged to remove any reagent stuck to the side or top of the tubes.

Unknown and QC (three levels L, M, H in duplicate) samples were prepared by adding 200 μ L of water, 20 μ L of ammonium acetate @ pH 4 and 20 μ L of internal standard to appropriately labeled 1.5 ml snap top centrifuge tubes using an Eppendorf repeater pipette. 200 μ L of the unknown or QC sample was then pipetted into its respective tube and briefly mixed on a vortex mixer and pulse centrifuged to remove any reagent stuck to the side or top of the tubes.

2.7 Chromatography and detector conditions

Raltegravir was separated with a Shimadzu Shim-pack XR-ODSII 2.0 mm $\times$ 75 mm column. The separation was run at a column temperature of 50°C and samples were maintained at 15°C. The overall run time was 8 min at a flow rate of 0.6 ml min⁻¹. Mobile phase A consisted of 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The gradient was a starting mixture of 25 % B maintained for 1 min followed by a linear gradient from 25–90 % B over 4 min, followed by a 1 min 100 % B wash and a 2 min re-equilibration step. MS detection was performed with an API 5000 in MRM Positive mode, ESI. The Q1/Q3 was 445.2/361.2 for raltegravir and 451.1/367.2 for the internal standard. Other settings included and 80 msec dwell time, DP of 121, CE of 23, CXP of 50 CAD gas at 5.00 CUR gas at 30, GS1 at 55, GS2 at 55, IS of 4800, EP of 10 and Temp of 600 °C.

2.8 Calibration curve and concentration determination

The calibration curves were determined by plotting the theoretical concentration vs. the area ratio for each standard. A weighted ($1/x^2$) linear regression line was fit to the data and used to determine the concentration of raltegravir on each day. In order for the calibration curve to be acceptable at least 14 of 18 (75%) of the back calculated standard concentrations had to vary $\leq$ 15% from the theoretical target. At the LLOQ the allowable error is $\leq$ 20%. If data points were dropped from the curve, the curve was recalculated. These dropped data points were not used in calculations.

2.9 Analytical method validation

Initial validation was performed according to the FDA guidelines for industry [9]. Validation included assessment of the standard curve performance, intra- and inter-day assay precision and accuracy, analyte stability, dilution effects, determination of potential matrix effects and the determination of specificity.

2.10 Accuracy and precision

Intra- and inter-day accuracy and precision for the assay was determined over five days with five sample replicates at four different concentrations of raltegravir spiked into PBMC extract. These concentrations were: the lower limit of quantification (LLOQ) (0.0023 ng ml⁻¹), a low standard ($\geq 3 \times$ the LLOQ, 0.0069 ng ml⁻¹), a medium standard (0.074 ng ml⁻¹) and a high standard ($\geq 80\%$ of the upper limit of quantification (ULOQ), 7.4 ng ml⁻¹). Accuracy was determined by calculating the percent deviation (%Dev) from the expected concentration. Precision was calculated by determining the coefficient of variation (%CV).

2.11 Dilution effects

Human PBMC extract was spiked at a concentration of 27.6 ng ml⁻¹, a concentration well above the ULOQ of the assay. In order to determine the concentration of this sample, a dilution must be made so the final concentration would be less than 10 ng ml⁻¹. Three replicates of three dilutions were made and extracted: (1) 1:10 (100 μ L sample + 900 μ L blank cell extract); (2) 1:20 (50 μ L sample + 950 μ L blank cell extract); (3) 1:40 (25 μ L sample +

975 μl blank plasma). The resulting theoretical concentrations of these dilutions are 0.69, 1.38, and 2.76 ng ml^{-1} .

2.12 Stability

Low (0.0069 ng ml^{-1}) and high (7.4 ng ml^{-1}) validation samples were subjected to various conditions in order to test analyte stability including freeze/thaw, exposure to room temperature, and extracted samples kept in the sample carrier for an extended period of time.

2.13 Freeze/Thaw Stability

Validation samples were frozen at $-80\text{ }^{\circ}\text{C}$ for a minimum of 24 hours, then thawed completely and allowed to remain at room temperature for 12 to 24 hours. This freeze/thaw cycle was repeated three times. These validation samples were then extracted in triplicate and compared with a triplicate preparation of validation samples that had not been subjected to this freeze/thaw cycle.

2.14 Room temperature stability

Validation samples were allowed to remain at room temperature for 24 and 96 hours. These validation samples were then compared with a triplicate preparation of validation samples that had been allowed to equilibrate to room temperature immediately prior to analysis.

2.15 Reinjection stability

Validation samples were extracted and assayed as normal. Following the initial run, the samples were allowed to remain in the auto-sampler compartment at $15\text{ }^{\circ}\text{C}$ for 24 hours and an extended time of six days. After this time, the samples were re-injected and compared with the previously obtained data.

2.16 Long-term stability

Validation/QC samples stored at $-20\text{ }^{\circ}\text{C}$ were compared with the initial time point. Data for up to five months has been collected and no apparent degradation of the QC with time as exhibited by the lack of a significant slope or correlation was observed.

2.17 Stock solution stability

Analyte stock solutions were prepared in methanol at a concentration of 1 mg ml^{-1} . Immediately following preparation, in order to determine a control for stability, the stock solution was diluted in triplicate, with 50% acetonitrile to $10\times$ the indicated concentration. These were then treated as standards and yielded the indicated results. A portion of the stock solution was left at room temperature for 24 hours and assayed in the same manner as above and compared with the initial time point data.

2.18 Matrix effects

The isotopic internal standard and the analyte of interest (RAL) co-eluted at 2.73 minutes. Matrix purity, recovery, process efficiency and cell number variability were tested. This was necessary to include when employing MS technology because it is increasingly evident that due to matrix effects, MS-MS detection can potentially be less selective than previously thought. To assess matrix effects, five different blank PBMC lots were extracted and tested for environmental contamination and endogenous interferences.

2.19 Process efficiency (PE)

In order to test PE two different sets of samples were prepared. Set 1 consisted of un-extracted analyte with no matrix. This set defines the overall system and detector

performance, and specifically, consisted of no processing and simply raltegravir and internal standard spiked in 70% MeOH. Set 2 consisted of analyte spiked into matrix and then extracted. This set of samples defines recovery and overall process efficiency. Five different lots of PBMC extract were tested at low ($0.0069 \text{ ng ml}^{-1}$), medium (0.074 ng ml^{-1}), and high (7.4 ng ml^{-1}) concentrations for a total of 30 samples. Completed data sets with raltegravir areas, IS areas, and peak area ratios (PAR) for all five lots at the three concentration levels for both experimental sets were prepared. Mean, standard deviation, and coefficient of variance were calculated across the five different lots. Variance, PE ratios and slopes of standard lines were compared and analyzed to check for possible matrix effects.

2.20 Selectivity

Multiple antiviral drugs were tested for potential interaction with raltegravir. These analytes were combined with raltegravir to a final concentration of 10 ng ml^{-1} of each separate antiviral and 0.074 ng ml^{-1} raltegravir, and the results determined. These antiviral agents included maraviroc, emtricitabine, tipranavir, nevirapine, abacavir, indinavir, lamivudine, stavudine, zidovudine, nelfinavir, amprenavir, delavirdine, saquinavir, didanosine, darunavir, etravirine, tenofovir, efavirenz, atazanavir, ritonavir and lopinavir.

2.21 Patient derived extracts

Cells from patients, who were being treated with raltegravir and gave their informed consent were isolated with two cell preparation tubes and re-suspended in the upper layers of the tube. These cells were combined and counted using a Countess cell counter. The cells were split into two, 15 ml centrifuge tubes and spun to remove them from the plasma. The plasma was carefully removed and discarded. Ten ml of ice cold PBS were added to one tube to re-suspend the cells. These cells were spun 10 min at $400 \times g$ to remove the cells and all the PBS was discarded except for about 1 ml. The cells were suspended in the residual 1 ml of PBS and transferred to a fresh 1.5 ml centrifuge tube and spun for an additional 10 min at $400 \times g$. The supernate was removed and the cells were suspended in 500 μl of cold 70% MeOH. This and similar methods employing a cell wash are commonly used for cell preparation and extraction for measuring intracellular NRTI and has been used to measure raltegravir [8, 10, 11, 12].

To the other tube of cells, 1 ml of cold PBS was added to re-suspend the cells. The suspension was carefully layered onto 150 μl of Nyosil M25 oil. The cells were spun through the oil at $17,000 \times g$ for 1 min at 4°C . The PBS was removed and the space above the oil washed twice with 1 ml of water. The water and oil was removed and the cells extracted with 500 μl of cold 70% MeOH.

In both cases an aliquot of the extract corresponding to 2 million cells was diluted to 200 μl and treated as described above for determining intracellular raltegravir in unknown samples. The results are expressed as fmol (million cells) $^{-1}$.

3. Results and discussion

3.1 Standard curve performance

All nine of the standard concentrations were run in duplicate on five separate days. The experimental standard concentrations were back calculated using the resulting regression curve for each day. The resulting data are shown in Table 1 (Inter-day Average Back Calculated Calibration Standards). The data in Table 1 represent the means of the five days of data. Precision and accuracy was excellent across all concentrations, with the greatest average absolute %CV and percent deviation less than 7.52% and 4.96%, respectively. The

R^2 values were all 0.996 or above indicating good linearity. Figures 1 and 2 shows the intensity of the assay at the upper and lower ends of the curve as well as the internal standard response at the same concentration. Figure 3 is an example of a blank spiked with internal standard showing the internal standard does not produce any signal in the assay channel. Therefore, it was determined the regression curves obtained can be used to accurately and precisely determine unknown sample concentrations.

3.2 Accuracy and precision

Accuracy and precision were tested both within each run (intra-assay) and between each run (inter-assay) by calculating the average percent deviation from the theoretical concentration and by calculating the %CV. Table 2 shows all inter-assay variability was within tolerance of less than 20% at the LOQ and less than 15% at the other points. As expected, the most variability was at the LOQ with an overall CV of 17.85%. The %CV and %deviation were 11.27% or less and -4.13% or less at the low, medium and high validation points. This indicates that 0.0023 ng ml⁻¹ represents a number that is near the limit of quantitation for this assay. Table 3 indicates the daily variability over the five days of the assay or the intra-assay variability. The % CV and % deviation values were again less than 20% at the LLOQ. At the other concentrations all % CV and % deviation values were less than 15%.

3.3 Sample dilution and stability studies

Table 4 shows sample dilution resulted in predictable concentrations with a deviation 5.36% over a range of dilutions from 1:10 to 1:40. The effect of three freeze thaw cycles was minimal at 3% difference from control with both high and low validation samples. Stability in the sample chamber (15°C) was excellent with an absolute difference 4.03% over 6 days tested with high and low concentrations of validation sample. Initial extracted sample stability at room temp was tested with high and low concentrations of validation and was acceptable with 5.93% change after 96 h. This was to show that the samples would still be viable if there was an equipment failure resulting in exposure to room temperature for an extended period of time. The stability of the stock solution varied 7.0% over a 24 period left at room temperature.

3.4 Potential matrix effects

Across five different matrix preparations the most variability was seen when no matrix was present at 7.25 % (Table 5a) indicating the variability was due to experimental rather than matrix variation (Table 5a). There was less than 10% variability in peak area ratios (PAR) (analyte areas/internal standard areas). Process efficiency measures as shown in table 5b confirmed good recovery and process efficiency (Table 5b). In addition the slopes of the lines constructed with the apparent concentrations were consistent with a %CV 3.6% (Table 5c) confirming the absence of any matrix effects.

3.5 Assay specificity

All of the antiviral compounds tested showed <10% difference from the blank when a 10 ng ml⁻¹ solution of each was combined with a solution of raltegravir to produce a final concentration of 0.074 ng ml⁻¹. The % difference from blank varied from -6.17 to 9.15, with tipranavir and nelfinavir producing the largest deviations at 9.15 and 8.71, respectively.

3.6 Patient derived samples

One of the concerns with the determination of intracellular raltegravir was potential sample loss due to diffusion of raltegravir out of the cells during processing. We used a novel approach of a rapid spin through oil to process our raltegravir-containing patient samples. We, and other groups have used this method for in vitro experiments where rapid separation

of the cells from media or treatment drug is desired [13–17]. Processing time is minimized by spinning the cells through mineral oil to quickly isolate the cells from the aqueous phase or in our case the plasma. The oil acts as a barrier to the aqueous phase, which can be removed, and the area above the oil washed to remove any residual parent drug [13,16]. In our case this technique was used to minimize processing time as the cells were rapidly extracted after centrifugation through oil. Thirty-five samples were collected from 17 HIV-infected persons who were receiving raltegravir. Each sample was prepared using the two different sample processing methods described above. In every patient sample there was an appreciable increase in apparent intracellular raltegravir concentration when processed through oil. The mean increase was 51% with a range from 16.9% to 156.8%. The mean, median, minimum and maximum intracellular raltegravir concentrations when processed by the conventional method were: 120.7, 34.3, 0.7 and 1473.1 fmol 10^6 cells⁻¹, respectively (188.5, 53.6, 1.1, and 2300.6 ng ml⁻¹, respectively, using 282.9 as the cell volume) [18]. The mean, median, minimum and maximum intracellular raltegravir concentrations when processed through oil were: 172.1, 53.9, 1.0 and 1984.8 fmol 10^6 cells⁻¹, respectively (268.8, 84.2, 1.6, 3099.8 ng ml⁻¹, respectively). Centrifuging the cells through an oil layer resulted in a significant increase ($P=0.002$, paired t-test) in apparent intracellular raltegravir concentration.

4. Conclusions

This analytical procedure for measurement of intracellular raltegravir concentrations from human cell extracts is accurate and highly sensitive with a lower limit of quantitation of 0.0023 ng ml⁻¹. Assay sensitivity is more than adequate for quantification of raltegravir concentrations in plasma from patients taking the usual dose where trough concentrations have been shown to range from 15 ng ml⁻¹ to 1800 ng ml⁻¹, and for quantification of intracellular concentrations of raltegravir in human PBMCs as shown in the present work. The method we developed for processing cells from patients rapidly through oil yielded consistently higher intracellular concentrations of raltegravir compared with the conventional method that includes a cell wash. The increased concentrations of raltegravir with the oil technique is thought to be the result of reduced diffusion of the raltegravir due to the more rapid time to extraction and the omission of the time-consuming wash steps. In addition to requiring more time, the wash also provided a relatively large volume for the intracellular raltegravir to diffuse into. This reasoning has also been proposed by other investigators using different techniques [19]. However, at this time we cannot rule out the possibility the reduced concentration in the conventional procedure is the result of cell loss compared with the oil methodology. In addition to emphasizing the importance of isolation technique, this method allows new insights into the exposure-response profile for raltegravir because it demonstrates that intracellular raltegravir concentrations in patients are higher than previously reported in the literature.

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Highlights

- The assay for raltegravir was highly sensitive with an LLOQ of 0.0023 ng/ml.
- The LLOQ was 40 to over 100 fold more sensitive than assays in the literature.
- A novel, rapid method of cell processing was developed and used in this study.
- This method resulted in 40% greater intracellular raltegravir concentrations.

Figure 1.
Intensity of the assay at the lower limit of quantitation
A – Analyte intensity
B – Internal standard intensity

2a

b

Figure 2.
Intensity of the assay at the upper limit of quantitation
A – Analyte intensity
B – Internal standard intensity

3a

b

Figure 3.
Intensity of the assay with a blank
A – Analyte intensity
B – Internal standard intensity

Table 1

Standard curve reproducibility data.

Raltegravir Concentration ng ml⁻¹	Mean Assayed Concentration ng ml⁻¹	% CV	% Deviation	n
0.0023	0.0024	5.47	2.18	8
0.0046	0.0044	5.97	-3.91	7
0.0092	0.0091	7.52	-1.23	8
0.046	0.0467	4.66	-4.43	9
0.092	0.0882	4.89	-4.18	10
0.46	0.4589	4.39	-0.25	10
0.92	0.9189	4.05	-0.33	10
4.6	4.828	3.93	4.96	10
9.2	9.519	6	3.47	9

Table 2

Inter assay Precision and Accuracy

	LLOQ	Low QC	Mid QC	High QC
Theoretical conc. (ng ml ⁻¹)	0.0023	0.0069	0.074	7.4
mean	0.0024	0.0066	0.0743	7.2676
SD	0.0004	0.0007	0.0063	0.5676
%CV	17.85	11.27	8.41	7.81
%dev	6.38	-4.13	0.44	-1.79
n	22	25	25	25

Table 3

Intra-assay variation

Run ID	Intraassay Statistics	LLOQ	Low	Medium	High
1	Theoretical				
	Mean(ng ml^{-1})	0.0023	0.0069	0.074	7.4
	SD	0.0020	0.0062	0.0742	7.3604
	%CV	7.94	11.68	4.55	1.51
	%dev	-14.60	-10.42	0.25	-0.54
2	n	4	5	5	5
	Mean(ng ml^{-1})	0.0024	0.0061	0.0837	7.8082
	SD	0.0004	0.0006	0.0046	0.4586
	%CV	16.18	10.55	5.54	5.87
	%dev	3.15	-11.85	13.11	5.52
3	n	4	5	5	5
	Mean(ng ml^{-1})	0.0027	0.0070	0.0707	6.3964
	SD	0.0005	0.0009	0.0061	0.4752
	%CV	18.79	12.79	8.65	7.43
	%dev	16.36	1.97	-4.53	-13.56
4	n	4	5	5	5
	Mean(ng ml^{-1})	0.0024	0.0068	0.0711	7.4210
	SD	0.0004	0.0006	0.0026	0.1204
	%CV	14.88	9.39	3.63	1.62
	%dev	6.21	-1.28	-3.95	0.28
5	n	5	5	5	5
	Mean(ng ml^{-1})	0.0027	0.0070	0.0720	7.3518
	SD	0.0004	0.0004	0.0033	0.3268
	%CV	15.28	5.95	4.58	4.44
	%dev	17.91	0.91	-2.66	-0.65
	n	5	5	5	5

Table 4**Dilution Study**

Target conc (ng ml ⁻¹)	27.6	27.6	27.6
Dilution	1:40 dil	1:20 dil	1:10 dil
Multiplication factor	40	20	10
Apparent Concentration (ng ml ⁻¹)			
Replicate			
1	0.7413	1.394	2.796
2	0.7028	1.402	2.957
3	0.6755	1.441	2.971
Corrected Concentration (ng ml ⁻¹)			
Replicate			
1	29.65	27.88	27.96
2	28.11	28.04	29.57
3	27.02	28.82	29.71
Mean	28.26	28.25	29.08
SD	1.322	0.503	0.972
%CV	4.68	1.78	3.34
%Dev	2.40	2.34	5.36
n	3	3	3

Table 5

Matrix effects tests

A - Precision of the indicated parameter across 5 different matrix lots						
Target Conc (ng ml ⁻¹)	Analyte Peak Area		Internal Std Peak Area		Peak Area Ratio	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
L 0.0069	7.25	5.6	0.99	3.29	7.16	5.88
M 0.074	3.28	3.53	1.87	2	3.5	2.66
H 7.4	2.6	1.68	4.98	2.22	3.58	1.69

B - Matrix Effect (ME) and Process Efficiency (PE)						
Target Conc (ng ml ⁻¹)	Analyte Peak Area		Internal Std Peak Area		Peak Area Ratio	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
L 0.0069	1121.6	1197	29009.7	28537.2	0.0387	0.0420
M 0.074	12661.7	12264.8	28392.3	27822.9	0.4460	0.4408
H 7.4	1253908	1231050	28962.4	28295.2	43.3480	43.5160

C - Comparison of the slopes of lines made from the three concentrations and the 5 different lots in set 1 and set 2 Slopes		
Matrix Lot #	Set 1	Set 2
1	5.3150	5.4145
2	5.6484	5.4829
3	5.1957	5.4408
4	5.3330	5.3035
5	5.5970	5.5538
Mean	5.4178	5.4391
SD	0.1952	0.0923
%CV	3.60	1.70