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Ultrasensitive Liquid Chromatography–Tandem Mass Spectrometric Methodologies for Quantification of Five HIV-1 Integrase Inhibitors in Plasma for a Microdose Clinical Trial

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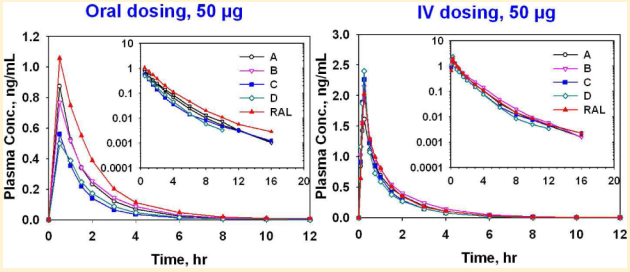
Supporting Information

ABSTRACT: HIV-1 integrase strand transfer inhibitors are an important class of compounds targeted for the treatment of HIV-1 infection. Microdosing has emerged as an attractive tool to assist in drug candidate screening for clinical development, but necessitates extremely sensitive bioanalytical assays, typically in the pg/mL concentration range. Currently, accelerator mass spectrometry is the predominant tool for microdosing support, which requires a specialized facility and synthesis of radiolabeled compounds. There have been few studies attempted to comprehensively assess a liquid chromatography–tandem mass spectrometry (LC–MS/MS) approach in the context of microdosing applications. Herein, we describe the development of automated LC–MS/MS methods to quantify five integrase inhibitors in plasma with the limits of quantification at 1 pg/mL for raltegravir and 2 pg/mL for four proprietary compounds. The assays involved double extractions followed by UPLC coupled with negative ion electrospray MS/MS analysis. All methods were fully validated to the rigor of regulated bioanalysis requirements, with intraday precision between 1.20 and 14.1% and accuracy between 93.8 and 107% at the standard curve concentration range. These methods were successfully applied to a human microdose study and demonstrated to be accurate, reproducible, and cost-effective. Results of the study indicate that raltegravir displayed linear pharmacokinetics between a microdose and a pharmacologically active dose.



Human immunodeficiency virus type 1 (HIV-1) is the virus that causes acquired immuno deficiency syndrome (AIDS), one of the world's leading causes of infectious disease related mortality.[1] Identified as one of the three essential HIV-1 enzymes, integrase is responsible for catalyzing the insertion of the viral DNA into the genome of host cells, a required step for HIV-1 replication.[2] Integrase inhibition has been pursued as a novel and unique antiretroviral mechanism that addresses the limitations with the established anti-HIV standard of care, particularly in patients with multidrug resistance. Raltegravir (Isentress, RAL, MK-0518), a potent, specific integrase strand transfer inhibitor, is the first and currently the only drug in this class approved for the treatment of HIV-1 infection.[3,4] Clinical phase I studies have demonstrated that RAL is generally well tolerated, at doses up to 1600 mg/day given for up to 10 days.[5] In healthy adults, RAL is absorbed relatively rapidly, with a median time to C_{max} (T_{max}) between 0.5 and 3.0 h in the fasted state. The mean terminal half-life is approximately 7–12 h at the therapeutic doses. Dose proportionality was displayed over the clinical relevant range of 100–800 mg. A human absorption, distribution, metabolism, and excretion (ADME) study suggested that the primary route of clearance is uridine diphosphate glucuronosyltransferase (UGT) 1A1 mediated glucuronidation.[6] Robust efficacy and a favorable safety profile were demonstrated in both treatment-naïve[7,8] and treatment-experienced[9] patients.

As an appealing approach in the drug development paradigm, microdosing is gaining growing interest since the pioneering human microdose pharmacokinetic study was conducted in 2003.[10] A microdose, by definition, is 1% or less of the pharmacologically active dose as projected from animal and/or *in vitro* models, not to exceed a maximum dose of 100 μg.[11,12] Given the extremely small quantities of drug substance administered, microdosing is considered safer and presents minimal risks for adverse and side effects. Regulatory requirements for preclinical safety testing and the demand for drug substance synthesis quantities are considerably lower in comparison with traditional phase I first-in-human studies at pharmacological doses.[13] Conceivably, this approach offers the opportunity to derive clinical relevant insights more rapidly, resulting in possible reductions to the drug development cycle time and cost. The mainstay of microdosing is for acquiring exploratory information on human pharmacokinetics (PK) for drug candidate selection.[10,14] Recently, its use has extended to

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studying pharmacodynamics, distribution, metabolism, drug−drug interactions, and biomarkers.[15−18]

A microdosing study presents daunting challenges for commonly used analytical techniques. In contrast to the pharmacological dose studies for which analyte concentrations typically fall within a ng/mL to μg/mL range, microdose studies often require measurements of analytes at low pg/mL or even subpg/mL concentrations. Accelerator mass spectrometry (AMS), a mass spectrometric technique based on measuring total radioactivity, emerged as a bioanalytical tool for quantifying radio-labeled compounds (most often with ^{14}C) in biofluids. Its absolute sensitivity can reach as low as subattomoles (10^{-18} mol),[19] and subpicomolar to femtomolar in biofluids.[20] Owing to its exquisite sensitivity, AMS has become the predominant analytical tool for microdose study support.[21] However, this technique requires specialized facility and radio-labeled compound synthesis, leading to a considerable cost compared to standard methodology. Other limitations include the lack of specificity in distinguishing parent drug and metabolites and tedious sample preparation procedure.

Liquid chromatography (LC) coupled with triple quadrupole MS is widely adopted as the tool of first choice for quantitative bioanalysis, with a typical working range in the ng/mL level.[22] Continued theoretical and technical advances in areas of sample preparation, LC, and mass spectrometry[23−25] have enabled significant progress toward applying conventional LC−MS/MS to clinical microdosing applications.[26−30] The major advantages of using an LC−MS/MS approach reside in reduced cost in bioanalysis and, more importantly, the opportunity to conduct microdosing without the need for radio-labeled compound synthesis.[31] Notably, Yamane et al. investigated the quantification limits of 31 marketed drug compounds with diverse structures, and the study results supported the viability of the LC−MS/MS approach for 30 out of 31 drugs.[32] Also implicated in Yamane et al.'s study is that the feasibility of lowering quantitation limits to pg/mL may vary dramatically from compound to compound. For example, the LLOQ of the 30 compounds spread between 0.08 and 50 pg/mL, using liquid−liquid extraction (LLE) or solid-phase extraction (SPE) followed by LC−MS/MS analyses. For the few compounds that are readily ionizable into the gas phase, it may be possible to develop ultrasensitive assays following standard LC−MS/MS approaches; while for others, a more complex analytical workflow may be entailed.

For ultratrace analyses, analyte enrichment and sample cleanup using SPE[33,34] and column switching[35] helped achieve pg/mL quantitation limits. Ultraperformance liquid chromatography (UPLC) provides superior separation efficiency which may translate into improved detection limit.[36−38] Downscaling the conventional LC system, i.e., to nano-LC, reduces dilution of the analyte zone, hence, offers opportunities for sensitivity gain, especially when online preconcentration is applied in combination to overcome injection volume limitation on nano-LC systems.[39,40] Nanoelectrospray promises inherently higher efficiency in ionization and ion transmission than that of conventional electrospray, attributed to the smaller size initial droplets under nanoflow conditions.[39] Chemical derivatization and mobile phase additives can facilitate analyte ionization via installing readily ionizable moieties to a certain molecular structure or formation of analyte-additive adduct ions.[42−44] Potentially, these approaches can help overcome the sensitivity limitations of LC−MS/MS based methods; however, few studies have been attempted to assess them in the context of microdosing applications.

In an ongoing drug discovery program, four proprietary compounds (A, B, C, and D) were identified as potent and selective integrase inhibitors. This series of compounds is difficult to synthesize. Furthermore, on the basis of studies of other drug candidates, predictability of human PK characteristics using preclinical *in vitro* and *in vivo* models is poor. To aid candidate selection and prioritization for clinical development, a microdose pharmacokinetic study was warranted. In this study, RAL and the four investigational compounds were dosed at 50 μg both orally and intravenously, with RAL being a benchmark against which the PK parameters of the new compounds were compared. In order to adequately characterize PK profiles of the five compounds, bioanalytical assay lower limits of quantitation (LLOQ) at low pg/mL was required.

This manuscript describes the development of ultrasensitive LC−MS/MS methods for the determination of five integrase inhibitors. This group of compounds is not intrinsically sensitive on MS. The approach to maximize assay sensitivity was to combine the strengths of multiple analytical techniques and use compound physicochemical properties as a guide for assay optimization. An assay LLOQ of 1 pg/mL was achieved for RAL and 2 pg/mL for the other four compounds. Notably, the RAL assay was at least 1000-fold more sensitive than the published methods to date.[45−48] To our knowledge, this is the first description of a microdose study for HIV-1 integrase inhibitors. The microdosing assay performance has been rigorously assessed and fully characterized in accordance with FDA guidances on clinical bioanalysis. In particular, intra- and inter-run performance at the assay LLOQ has been investigated, which is an essential aspect for the success of ultratrace analysis. The assays were implemented in support of a clinical trial in which approximately 1000 PK samples were analyzed, with results demonstrating high reproducibility and robustness. Our study suggests the feasibility of developing conventional LC−MS/MS methods with exceptional assay sensitivity (e.g., down to low pg/mL) that are suitable for clinical microdosing support.

EXPERIMENTAL SECTION

Chemicals and Reagents. Reference standards for RAL potassium salt (purity 99.2%) and stable isotope $^{13}C_6$-labeled RAL internal standard (purity 97.7%) were synthesized at Merck Research Laboratories. The structure of RAL is shown in Figure S-1 in the Supporting Information. The stable isotope labeled internal standard (IS) [$^{13}C_6$] RAL was synthesized with C-13 distributed over the six carbons within the phenyl ring bearing the fluoro group. The reference standards (purity ≥98.3%) and corresponding stable isotope $^{2}H_6$ labeled internal standards for the four proprietary compounds A, B, C, and D were also synthesized in-house. Control human plasma with K_2EDTA as the anticoagulant was purchased from Biological Specialty Corporation (Colmar, Pennsylvania). Formic acid, acetic acid, ammonium hydroxide concentrated solution (28−30% w/w), ammonium formate, acetonitrile, and methyl *t*-butyl ether (MTBE) were obtained from Fisher Scientific (Waltham, Massachusetts). All reagents and solvents were of analytical or HPLC grade.

Clinical Trial. The microdose study involved five parallel panels and six subjects per panel. The subjects enrolled in the study were healthy males between 18 and 45 years of age. Informed consent was reviewed and signed by each subject.

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Each panel was administered one of the five compounds at a 50 μg dose, orally in period 1 and intravenously in period 2. Within each panel, there was at least a 7-day washout between the two periods. Blood was collected into a plastic Vacutainer containing spray-dried K_2EDTA at predose, and 30 min, 1, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36, and 48 h postdose for period 1. For period 2, blood was collected at predose and postdose at 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36, and 48 h. Plasma PK samples were stored at −20 °C until analysis.

Solution Preparation. Stock solutions were prepared at a concentration of 1 mg/mL in 50/50 (v/v) acetonitrile/water for all compounds. Working solutions used in the preparations of standard and quality control (QC) samples were diluted from stock solutions with 50/50 (v/v) acetonitrile/water. For each assay, a working solution of the internal standard (IS) was prepared at a concentration of 2 ng/mL. All solutions were stored at −20 °C and brought to room temperature before each use. Plasma standards were prepared on the day of sample analysis by spiking the appropriate volume of working solutions into control human plasma with K_2EDTA anticoagulant. In each analytical run, control blank samples with and without the IS working solution spiked in were assayed to ensure lack of interferences from the control matrix and the IS. Plasma QCs were prepared by dilution of QC working solutions with control plasma and frozen at −20 °C until analysis.

Plasma Sample Preparation. The same sample preparation procedure, as described in the following, was applied for analyses of all five compounds. An aliquot of 900 μL of plasma sample and 25 μL of internal standard working solution were added to a 2-mL 96-well polypropylene plate on a Packard MultiPROBE robotic liquid handler (Perkin-Elmer, Waltham, Massachusetts), followed by acidification with 500 μL of formic acid (10% in water). After mixing, the sample mixture was then transferred by a Quadra 96 workstation (Tomtec, Hamden, CT) to a 96-well Waters Oasis HLB SPE plate with 60 mg of sorbent (Milford, MA) preconditioned with methanol and water. A slight vacuum was applied, as needed, for loading the samples onto SPE sorbent. Washing solvents in the sequence of 2% ammonium hydroxide in 5% methanol/95% water (1.6 mL), 5% methanol/95% water (1.6 mL), 2% formic acid in 5% methanol/95% water (1.6 mL), and 50% methanol/50% water (3.2 mL) were then applied. The analytes were eluted with 1 mL of acetonitrile into a 2-mL 96-well plate. The organic solvent was dried under a gentle stream of nitrogen at 37 °C. Then, the analytes were reconstituted into 200 μL of 100 mM ammonium acetate (pH 4.5), followed by liquid−liquid extraction with 1 mL of MTBE. After phase separation, the organic layer was transferred to a clean injection plate and dried under nitrogen at 37 °C. Finally, the sample was reconstituted in 50 μL of 30% acetonitrile/70% water. An aliquot of 5 μL of the sample extract was injected onto UPLC−MS/MS for analysis. The injection volume was increased to 10 μL under the circumstances when instrument sensitivity for a 5 μL injection was not adequate for achieving a signal-to-noise ratio of ≥10 at the LLOQ.

LC−MS/MS Conditions. The LC column for analysis of RAL and compound A and B was a Waters Shield RP18 (1.7 μm, 2.1 mm × 50 mm) (Milford, MA). For compounds C and D, a Waters BEH C18 column (1.7 μm, 2.1 mm × 50 mm) (Milford, MA) was used. For all compounds, the mobile phases A and B were 0.05% acetic acid in water and 0.05% acetic acid in acetonitrile, respectively, and the flow rate was set at 600 μL/min. Details of gradient elution conditions are provided in Table S-1 in the Supporting Information.

An AB Sciex API 5000 tandem mass spectrometer equipped with a turbo-ion spray source (Applied Biosystem/MDS, Ontario, Canada) was interfaced with a Waters Acquity UPLC system (Milford, MA). It was operated in negative ionization and selected reaction monitoring (SRM) mode for monitoring selected precursor and product ions for the analytes and internal standards. The optimized MS/MS parameters for the five compounds are listed in Table S-2 in the Supporting Information.

Data Analysis. Mass spectrometric data were acquired and processed using Analyst software version 1.4.2 (Applied Biosystem/MDS Sciex, Canada). Calibration curves were constructed by plotting the peak area ratios of analyte to IS versus analyte nominal concentrations. Analyte concentrations were calculated using a $1/x^2$ weighted linear least-squares regression analysis of the standard curve. Accuracy (%) was expressed by [(mean observed concentration)/(nominal concentration)] × 100. Precision (%) was calculated by [(standard deviation)/(mean observed concentration)] × 100. Pharmacokinetic parameters were determined using noncompartmental analysis in WinNonLin (Pharsight, Mountain View, California). Nominal sampling times were used for PK calculations.

RESULTS AND DISCUSSION

The triple quadrupole mass spectrometer is a powerful and versatile tool widely used for targeted quantitative bioanalysis.

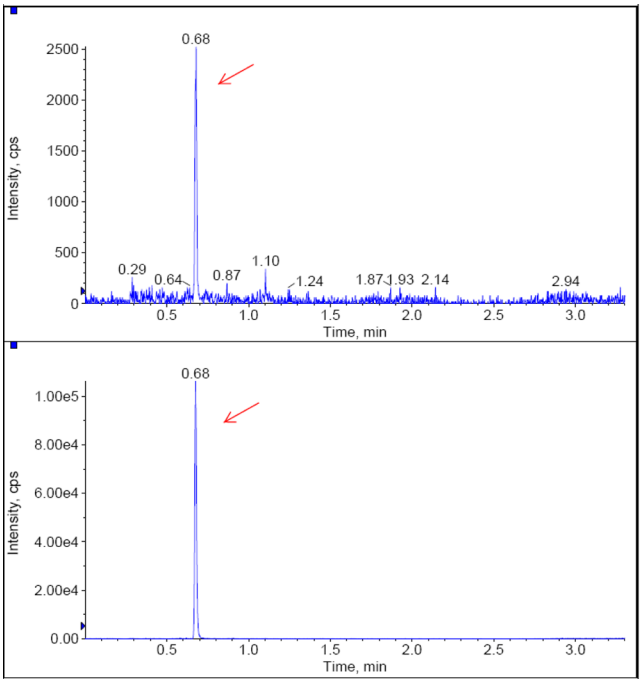

Figure 1. Extracted ion chromatogram for 1 pg/mL RAL extracted plasma sample. Upper panel, analyte; lower panel, IS.

Its selected reaction monitoring (SRM) capability allows detection of structure specific ion transitions and offers remarkable selectivity and sensitivity. Electrospray is a "soft" ionization technique well suited for analysis of small molecules with moderate polarity. The highly complex nature of matrix components in biofluids often adversely impacts ESI-MS assay

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Table 1. Intra- and Inter-Run Accuracy and Precision at Lower Limit of Quantitation (LLOQ)

compound	raltegravir	A	B	C	D
standard curve range (pg/mL)	1−1000	2−2000	2−2000	2−2000	2−2000
accuracy range (%)[a]	94.2−106	95.3−104	96.7−103	96.2−107	93.8−107
precision range (%)[a]	1.33−14.1	1.39−4.57	1.20−10.5	2.67−7.11	1.72−10.8
N	6	6	6	6	6
nominal QC concn (pg/mL)	1, 3, 75, 750	2, 6, 150, 1500	2, 6, 150, 1500	2, 6, 150, 1500	2, 6, 150, 1500
intrarun variability					
accuracy range (%)[b]	85.6−100	91.8−102	93.1−104	92.8−107	95.9−108
precision range (%)[b]	1.36−13.9	1.35−10.3	1.17−13.1	1.74−9.56	3.33−6.31
N	5	5	5	5	5
inter-run variability					
accuracy range (%)[b]	91.7−101	97.2−102	97.2−107	98.5−105	96.6−103
precision range (%)[b]	3.67−6.08	1.95−6.38	3.15−8.59	1.86−7.28	1.68−4.21
N	8	10	8	8	10

[a]Standard curves were prepared in plasma from six different sources. [b]QCs prepared at LLOQ concentrations (N = 5) were tested on 3 different days.

Table 2. Difference from Control (%) during Analyte Stability and Reinjection Reproducibility Assessment

nominal concn (pg/mL)	3 freeze (−20 °C)−thaw cycles	ambient temperature	processed sample	reinjection
Raltegravir				
3	−8.3	−4.6[a]	−2.0[d]	0.0
75	−3.4	−2.6[a]	−3.5[d]	−2.8
750	−2.8	−3.6[a]	−0.3[d]	−0.4
Compound A				
6	−2.2	−1.4[b]	−0.5[d]	−0.7
150	−0.2	5.5[b]	−0.4[d]	1.9
1500	0.1	−0.1[b]	−1.9[d]	0.5
Compound B				
6	−1.8	−1.9[a]	−1.4[e]	−3.9
150	−0.1	0.0[a]	−0.7[e]	0.2
1500	0.5	0.1[a]	−0.7[e]	0.3
Compound C				
6	−0.6	−2.6[c]	−9.8[d]	−2.8
150	−4.2	2.2[c]	1.1[d]	2.0
1500	1.4	0.6[c]	−2.1[d]	−1.5
Compound D				
6	−1.1	4.8[a]	1.0[f]	0.2
150	−0.4	−0.7[a]	2.6[f]	1.8
1500	−1.6	−0.4[a]	1.2[f]	0.5

[a]T = 24 h. [b]T = 17 h. [c]T = 5 h. [d]T = 3 days. [e]T = 2 days. [f]T = 4 days.

sensitivity, selectivity, and reproducibility. Thus, the importance of sample preparation and LC separation should be emphasized, especially for ultratrace analysis that needs analyte enrichment for bringing the concentrations of target analyte into the MS detectable range. We aimed at developing and validating five LC−MS/MS-based methods for determination of the integrase compounds in human plasma at pg/mL concentrations, using the aforementioned techniques which are widely available in a regulated bioanalysis laboratory setting.

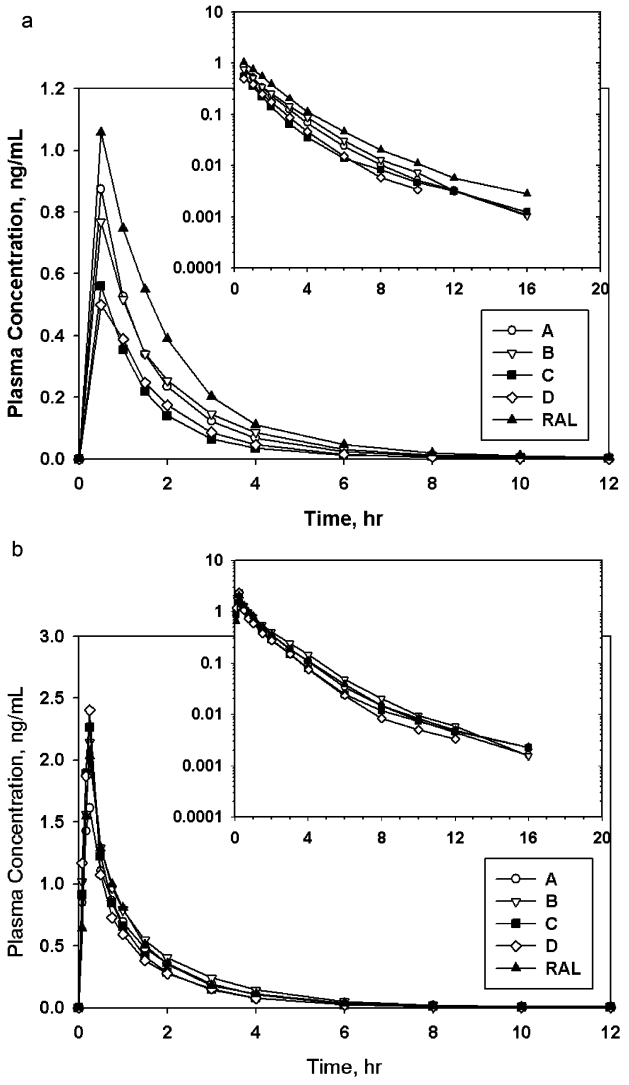

Figure 2. Mean concentration−time profiles (linear and log scale) following 50 μg of (a) oral dose and (b) IV dose.

Method Development. *Preliminary Evaluation and Analytical Challenges.* This series of integrase inhibitors belongs to the pyrimidinone structural class, as illustrated in Figure S-2 in the Supporting Information (The full structures cannot be shown for proprietary reasons). They are moderately hydrophobic, with a log D of approximately 2 at pH 7. Illustrated in Figure S-1 in the Supporting Information are the product ion spectra for RAL under the positive and negative ion modes. Apparently, the positive ion mode yielded higher signal intensity. Leveraging previous experience with RAL,[43−48] the preliminary method development endeavor focused on a standard SPE procedure for sample cleanup and analyte enrichment, followed by reversed phase UPLC separation using columns packed with 1.7 μm particles and positive ion ESI-MS/MS detection.

Compound A was used as a model compound for method development. Considering the structure similarity, assay conditions for the other four compounds were anticipated to be similar. An aliquot of 1.0 mL of plasma was extracted and reconstituted into 100 μL of solution after extraction, resulting in a 10-fold analyte preconcentration. The pH and solvent

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Table 3. Summary of PK Parameters[a]

compd	route of administration, dose (μg)	$AUC_{0-\infty}$ (nM h)	F	C_{max} (nM)	T_{max}[d] (h)	terminal $t_{1/2}$[e] (h)	CL (L/h)	V_{dss} (L)
A	PO, 50	2.69 (21)	0.57 (7)[b]	1.76 (21)	0.50 (0.50, 0.50)	3.02 (0.95)		
	IV, 50	4.37 (18)		3.32 (14)	0.25 (0.17, 0.50)	3.30 (1.07)	23.4 (18)	40.3 (16)
B	PO, 50	2.62 (41)	0.54 (29)[b]	1.49 (38)	0.50 (0.50, 1.00)	2.28 (0.48)		
	IV, 50	5.20 (23)		4.23 (30)	0.25 (0.25, 0.25)	2.75 (1.07)	19.7 (23)	34.3 (13)
C	PO, 50	1.60 (26)	0.43 (13)[b]	1.08 (24)	0.50 (0.50, 1.00)	3.31 (1.57)		
	IV, 50	4.12 (10)		4.31 (21)	0.25 (0.25, 0.25)	4.08 (2.11)	23.5 (10)	38.1 (22)
D	PO, 50	1.75 (18)	0.53 (14)[c]	1.03 (14)	0.50 (0.50, 1.00)	1.69 (0.26)		
	IV, 50	3.96 (18)		4.59 (37)	0.25 (0.25, 0.25)	2.22 (0.26)	25.1 (18)	32.5 (24)
RAL	PO, 50	4.37 (30)	0.79 (11)[c]	2.26 (46)	0.50 (0.50, 1.00)	4.29 (4.42)		
	IV, 50	5.22 (23)		4.48 (25)	0.25 (0.25, 0.25)	3.40 (0.42)	21.5 (23)	34.2 (14)

[a]F, bioavailability (fraction), adjusted for actual dose; V_{dss}, estimated volume of distribution at steady state; CL, clearance. [b]Geometric mean (% CV), n = 5. [c]Geometric mean (% CV), n = 3. [d]Median (min, max). [e]Harmonic mean (pseudo SD). [f]CL and V_{dss} calculated using the nominal dose of 50 μg.

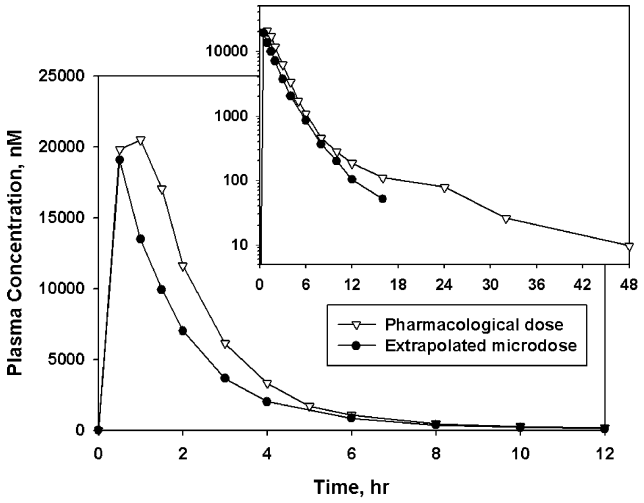

Figure 3. Mean concentration−time profiles (linear and log scale) for RAL following oral pharmacological dose (400 mg of oral suspension) and microdose. Microdose profile presented was normalized for a dose of 400 mg.

strengths for SPE washing and elution conditions were optimized to ensure analyte recovery and to remove matrix components with different physicochemical properties (e.g., hydrophobicity, polarity, acidity, or basicity, etc.). The chromatogram of an extracted 10 pg/mL of compound A plasma sample is shown in Figure S-3a in the Supporting Information. It appeared the chemical noise was quite high (up to several thousand counts) and interfered with the analyte quantification. Additionally, the preliminary assay suffered from issues such as severe matrix effect, elevated column back pressure over time, and poor reproducibility. Apparently, in this case, the performance of a standard LC−MS/MS approach was inadequate for quantifying low pg/mL concentrations in plasma.

Positive versus Negative Ion ESI. On the basis of ESI mechanisms by which gas phase ions are produced, analyte MS response is largely determined by parameters associated with the ionization and evaporation of analytes as well as their interactions with the electrolytes and solvents coexisting in the charged droplets generated at the electrospray tip.[49] High chemical background and matrix effect are attributed to the highly complex matrix components present at tremendously high concentrations (often orders of magnitude greater than those of the analytes) and/or those with very high MS responses. Switching to negative ion mode had the potential to yield cleaner ion chromatograms by eliminating the molecular species that do not form stable negative ions, such as those containing basic nitrogens and metal adducts. This group of integrase inhibitors is weakly acidic (pK_a at around 6.4), likely due to the contribution of the −OH group at the hydroxypyrimidinone core. This allowed negative ion detection of the compound, under which the background noise was reduced by a factor of more than 10-fold than that in the positive ion mode, without resorting to any extraction or chromatographic separation methods (Figure S-3b in the Supporting Information). Evidently, compound A yielded lower ion intensity when in negative mode than in positive. In an effort to enhance the analyte negative ion signal, the effect of additives in the mobile phases was studied. Formic acid or acetic acid, which helped achieve satisfactory chromatography, was added to methanol−water or acetonitrile−water-based mobile phases, and signal intensities for compound A were tested. The mobile phases with acetic acid added to acetonitrile−water manifested the highest signal intensity, showing 3-fold improvement compared with addition of formic acid. A plausible explanation is that the formation of analyte acetate adduct ions followed by leaving of the neutral acetic acid in the gas phase had facilitated production of the analyte ions. Similar "wrong-way round" electrospray ionization has been observed for other compounds bearing amide and other functional groups.[50,51] Interestingly, ion signal enhancement was not observed when methanol was used as the organic modifier of the mobile phase, indicating the significance of analyte−solvent interactions during ion generation.

Sample Extraction and Analyte Enrichment. This class of compounds lacks readily ionizable functional groups; thus, they do not form a high ESI-MS response. A more effective sample preparation methodology allowing analyte enrichment is a prerequisite for further lowering the assay LLOQ down to the targeted 1−2 pg/mL. Multiple sample extraction approaches were evaluated, including protein precipitation (PPT), LLE, supported liquid extraction (SLE), reversed phase SPE, ion exchange SPE, and hybrid-SPE-PPT. Evaluation results led to the conclusion that it is difficult to achieve the required sample cleanliness that would allow reliable measurements of the analyte at pg/mL concentrations by any single extraction technique available. A combination of multiple extraction mechanisms was necessary to add new dimensions of selectivity. Considering the factors of selectivity, sample cleanliness, and ease of automation, reversed phase SPE with

Waters Oasis HLB followed by LLE was found to be the best in addressing the assay needs. On the basis of a comparison of the analyte peak areas for reconstituted extracts of plasma samples prespiked with analyte and IS vs those spiked with analyte and IS at equivalent concentrations postextraction, the overall recovery was 50%. Matrix effect was evaluated by comparing the analyte peak areas in the extracted samples against those obtained in the neat reconstitution solution. A slight ion suppression of 15% was observed which was not expected to cause significant negative impact on assay performance. This double extraction procedure enabled an analyte enrichment factor of 18-fold without significant analytical issues. Assay throughput was maximized by a 96-well format extraction procedure amenable to automation.

Collectively, the approaches described above helped realize the detection of 2 pg/mL of compound A in plasma with a highly robust signal-to-noise ratio (Figure S-4a in the Supporting Information). Methods for the remaining four compounds were established following the same sample preparation procedure, and similar results were observed. The MS/MS response for RAL was greater than the other compounds. Therefore, an LLOQ of 1 pg/mL was established for RAL, while for the rest of the compounds, the LLOQ was 2 pg/mL. The ion chromatogram for RAL at 1 pg/mL (equivalent to 0.04 pg on column) are shown in Figure 1. Ion chromatograms for the other analytes are shown in Figure S-4 in the Supporting Information.

Method Validation. The five methods were fully validated in accordance with FDA guidance on regulated bioanalysis.[52−54] Assay linear calibration range was 1−1000 pg/mL for RAL and 2−2000 pg/mL for the remaining four investigational compounds. The observed linear regression coefficient (R) was ≥0.997 for all methods.

Assay LLOQ was established as the lowest concentration that can be quantifiable with precision ≤ 20% and bias ≤ ±20% of nominal. Both intra- and inter-run variability at LLOQ were within the acceptable range, with precision between 4.0 and 13.9% and accuracy between 85.6 and 107% of nominal (Table 1). No significant interfering peaks were observed at the time of analyte elution when examining blank human plasma from six different sources. Assay specificity in presence of potential concomitant medications were assessed by comparing the peak area ratios obtained from plasma samples spiked with the analyte and 10 commonly used over-the-counter (OTC) drugs (as listed in Table S-3 in the Supporting Information) with those from the control (spiked with analyte only). There was no significant difference (within 15%) observed for all five compounds. The major circulating metabolites in humans for this series of compounds were identified to be O-glucuronidation and demethylation products. Results from the study and poststudy incurred sample assessment suggested there was no observable metabolite interference during sample analysis or metabolite degradation during sample storage.

Within-run variability assessed by six replicates of standards prepared in plasma from six different sources demonstrated that the assay accuracy was between 93.8 and 107% and precision was between 1.2 and 14.1% at all concentrations of the curves (Table 1). Intrarun (n = 5) variability for plasma QCs prepared at low, middle, and high concentrations of the curve ranges were also assessed. Assay precision was within 5.33%, and accuracy was between 94.1 and 108%.

The analytes of interest were tested for stability in plasma when exposed to room temperature, storage temperature (−20 °C), or after 3 freeze−thaw cycles. No significant change in analyte concentrations was observed for the tested conditions (Table 2). Processed sample stability was established for up to 4 days, with no observable analyte degradation. Reinjection reproducibility was also confirmed for all assays, and the % change from the initial injection was within 4%.

Sample Analysis. A total of approximately 1000 clinical samples were analyzed for the microdose study, and 100% of the analytical runs passed the acceptance criteria. QC data from daily analytical runs demonstrated high assay accuracy and reproducibility (accuracy from 95.4 to 107%, precision from 1.68 to 7.38%). Analyte concentrations in plasma were quantifiable for up to 24 h postdose. Evidently, some of the concentrations at the terminal phases (e.g., those beyond 24 h) were not quantifiable with the present method LLOQ.

Theoretically, hyphenating nano-LC and nanoelectrospray increases the mass and concentration sensitivity.[39−41] In practice, this approach has demonstrated significantly improved detection limits, better tolerance for coeluting impurities, and reduced bias toward analytes with different liquid-to-gas transfer rates.[55−57] Therefore, for measurements of the below LOQ concentrations, a nanoscale LC−MS/MS approach is being investigated as a means for further sensitivity improvement.

PK Analysis Results. A fundamental hypothesis for the microdosing strategy is the predictability of PK following therapeutic doses from that following a microdose, namely, PK linearity. Three large studies were conducted in this regard.[58−60] In a recent review of all the drugs known for being tested in microdose clinical trials thus far, 80% of the drugs exhibited a difference of no more than 2-fold between PK parameters obtained from microdose and therapeutic dose.[17] On the basis of current understanding of the underlying molecular mechanisms, PK nonlinearity may arise as a result of poor solubility with solubility or dissolution rate limited bioavailability or a number of saturable kinetics involved in ADME, such as GI transporters, plasma protein binding/tissue distribution, first-pass effect, biliary/renal transporters, etc.[13]

Mean concentration−time profiles after an oral or intravenous 50 μg dose for the five compounds are depicted in Figure 2. The profiles revealed concentration declines from the peak in at least two exponential phases, somewhat similar to the observations in previous RAL PK studies.[5] PK parameters are summarized in Table 3. It should be noted that terminal half-life for the tested compounds may be underestimated due to the below LOQ values at the late terminal phase. RAL pharmacokinetics after 50 μg oral dosing was normalized for a therapeutic dose of 400 mg and compared with historical data following 400 mg oral suspension dosing. As illustrated in Figure 3, the PK profiles were similar, indicating linearity across the 8000-fold dose range. The PK linearity between a microdose and a clinically relevant dose for RAL supported human PK predictions for the investigational compounds using the microdosing strategy.

■ CONCLUSIONS

Microdosing study bioanalysis calls for ultrasensitive analytical methods that can reliably quantify analytes at pg/mL concentrations. Currently, AMS has been the mainstay bioanalytical technique in this domain. Compared with AMS, LC−MS/MS offers distinct advantages of cost effectiveness and allows the conduct of microdosing without the need of radiolabeled compound synthesis. However, LC−MS/MS has its

inherent technical issues that hamper its wide use in this field. Such issues include isobaric interferences, high chemical background, matrix effect, MS/MS detection limitation, etc. Hence, the application of LC−MS/MS for microdose bioanalysis is still at an early stage.

This manuscript describes the development of automated LC−MS/MS methods for five HIV-1 integrase inhibitors, with LLOQ at 1 or 2 pg/mL, for supporting a microdose clinical trial. Multiple separation and sensitivity enhancement mechanisms were used to achieve the desired sensitivity and selectivity. First, sensitivity was enhanced using analyte enrichment (by a factor of 18-fold), gradient elution on a UPLC column, and mobile phase additives that improved MS ionization efficiency. Second, high chemical background and matrix effect were reduced to a minimal level by operating under negative ion mode electrospray and performing effective sample cleanup via an optimized double extraction procedure. Furthermore, it was assured that these approaches worked in a concerted manner, which collectively made it possible to realize the extreme assay sensitivity. The five assays were fully validated to the standards of clinical regulated bioanalysis guidelines and implemented to analyze microdose plasma samples with high accuracy, reproducibility, and robustness. This study demonstrated an example of tackling the challenges presented in ultratrace analysis with the conventional LC−MS/MS approach and supports the use of LC−MS/MS as a feasible alternative to AMS in microdosing applications. PK linearity analysis for RAL indicates that the microdose study should provide reasonable predictions of PK parameters at clinically relevant doses for the investigational compounds being studied.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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