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Determination of an investigational HIV integrase inhibitor in human plasma using high performance liquid chromatography with tandem mass spectrometric detection

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Abstract

An HPLC–MS/MS assay for the determination of an HIV integrase inhibitor, 5-(1,1-dioxido-1,2-thiazinan-2-yl)-*N*-(4-fluorobenzyl)-8-hydroxy-1,6-naphthyridine-7-carboxamide (**I**) in human plasma has been developed and validated. Compound **I** and a stable isotope labeled internal standard (**II**) were isolated from 0.5 mL plasma samples by solid phase extraction using an Ansys SPEC C-8 96-well plate. Extracts were separated on a Hypersil BDS C-18 HPLC column (3.0 mm × 50 mm, 3 μm) with a mobile phase consisting of 25 mM ammonium formate pH 3.0:acetonitrile (60:40) vol%/vol% pumped at 0.5 mL/min. A Sciex API 365 mass spectrometer equipped with an atmospheric pressure chemical ionization source was operated in selected reaction monitoring (SRM) mode with the precursor-to-product ion transitions *m*/*z* 431 → 109 (**I**) and *m*/*z* 437 → 115 (**II**) used for quantitation. The assay was validated over the concentration range of 10–5000 ng/mL and was found to have acceptable accuracy, precision, linearity, and selectivity. The mean extraction recovery from spiked plasma samples was 69%. The intra-day accuracy of the assay was within 4% of nominal and intra-day precision was better than 4% C.V. Following a 200 mg dose of the compound administered to human subjects, concentrations of **I** ranged from 21.1 to 1500 ng/mL in plasma samples collected up to 12 h after dosing. Inter-day accuracy and precision results for quality control samples run over a 3-month period alongside clinical samples showed mean accuracies of within 6% of nominal and precision better than 3.5% C.V.

Keywords: HIV integrase inhibitor; Bioanalytical; HPLC–MS/MS

1. Introduction

Human immunodeficiency virus 1 (HIV-1), the virus that causes acquired immune deficiency syndrome (AIDS), requires 3 enzymes for its replication: protease, reverse transcriptase, and integrase. A number of antiviral agents that target protease or reverse transcriptase are currently on the market. These agents, typically administered in combination, have become standard therapy for the treatment of AIDS [1].

While the widespread use of protease and reverse transcriptase inhibitors has made a significant impact in improving survival rates of HIV infected patients, the emergence of drug-resistant HIV strains constitutes a growing problem in the treatment of AIDS. It has been estimated that over 76% of HIV infected adults in the United States harbor viral strains that are resistant to one or more of the antiviral drugs currently in use [2]. A need therefore exists for alternative antiviral therapies that are effective against these drug-resistant strains.

The HIV integrase enzyme catalyzes the incorporation of viral DNA into the genome of the host cell, and is thus essential to viral gene expression and replication [3]. Unlike protease and reverse transcriptase, an agent that targets the HIV integrase enzyme has yet to be marketed. Given its novel

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mechanism of action, an HIV integrase inhibitor has the potential to be highly effective in the treatment of AIDS, particularly for patients who have exhibited resistance to existing antiviral drugs.

Compound **I**, 5-(1,1-dioxido-1,2-thiazinan-2-yl)-*N*-(4-fluorobenzyl)-8-hydroxy-1,6-naphthyridine-7-carboxamide, is an HIV integrase inhibitor currently undergoing evaluation for the treatment of AIDS [4]. An assay for this compound was required to support clinical studies. The development of an HPLC–MS/MS assay for the determination of **I** in human plasma is the subject of this paper. To the authors' knowledge, this work is the first published report of the determination of an HIV integrase inhibitor in human plasma.

2. Experimental

2.1. Materials

Compound **I** (Fig. 1) was obtained from the Chemical Data Department of Merck Research Laboratories (Rahway, NJ, USA) in its sodium salt form. Compound **II**, a $^{13}C_6$ fluorophenyl analog of **I** (Fig. 1), was synthesized by the Drug Metabolism Labeled Compound Synthesis group of Merck Research Laboratories (Rahway, NJ, USA) using a variant of the synthetic route used to prepare **I** [5]. In this scheme, $^{13}C_6$ aniline was used to incorporate the labeled moiety into the molecule. Acetonitrile and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (95%) and ammonium formate were purchased from Sigma (St. Louis, MO, USA). Human control plasma (sodium heparin as an anticoagulant) was obtained from Sera Care Life Sciences (Oceanside, CA, USA). Deionized (18 mΩ/cm) water was generated in-house using a Millipore (Bedford, MA, USA) Milli-Q Plus system.

2.2. Instrumentation

The HPLC–MS/MS system consisted of a Varian ProStar 430 (Walnut Creek, CA, USA) autosampler, a Perkin-Elmer Series 200 (Norwalk, CT, USA) HPLC pump and a PE Sciex API 365 (Foster City, CA, USA) triple quadrupole mass spectrometer equipped with a atmospheric chemical ionization (APCI) source. Applied Biosystems-Sciex Analyst version 1.1 software was used for data acquisition and processing.

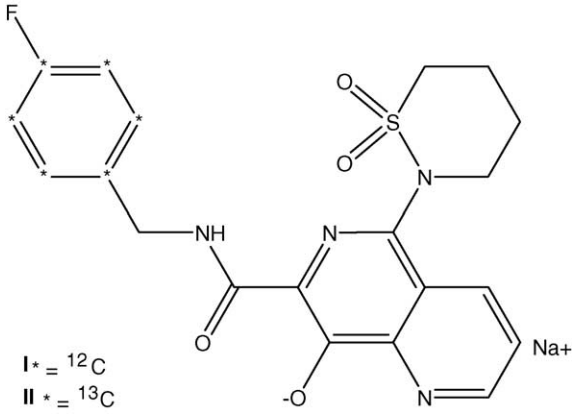

Fig. 1. Chemical structures of **I** and **II**.

2.3. Chromatographic conditions

The chromatographic separation was performed on a Hypersil BDS C-18 column (3.0 mm × 50 mm, 3 μm), which was purchased from Thermo-Hypersil Keystone (Bellefonte, PA, USA). A guard column (Hypersil BDS C-18, 3.0 mm × 10 mm, 5 μm) preceded the analytical column. The guard and analytical columns were thermostated at 30 °C. The mobile phase consisted of 25 mM ammonium formate pH 3.0:acetonitrile (60:40) vol%/vol% and was pumped at a flow rate of 0.5 mL/min. The pH of the aqueous buffer component of the mobile phase was adjusted with formic acid prior to mixing with acetonitrile. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane filter. The injection volume was 25 μL and the analysis time was 5 min per sample.

2.4. Mass spectrometer conditions

The effluent from the HPLC column was directed into an APCI probe, which was set to a temperature of 500 °C, a nebulizing gas (N_2) pressure of 60 psi, and a corona discharge needle current of 1.0 μA. The voltage applied to the sampling orifice of the mass spectrometer was 21 V. Protonated analyte molecules were subjected to collision induced dissociation using N_2 as the collision gas (CAD gas setting = 3) to yield product ions for each analyte. The collision energy was 35 V for both **I** and **II**. The voltages applied to the deflector and electron multiplier were −200 and −2400 V, respectively. Selected reaction monitoring of the precursor–product ion transitions *m/z* 431 → 109 for **I** and *m/z* 437 → 115 for **II** was used for quantitation. Dwell time was 300 ms for each ion transition. Product ion mass spectra for **I** and **II** are shown in Fig. 2.

2.5. Preparation of standard solutions

A 500 μg/mL (free phenol) stock solution of **I** was prepared by dissolving 5.25 mg of the sodium salt of **I** in approximately 5 mL of (50:50) vol%/vol% H_2O:CH_3CN in a 10 mL low actinic volumetric flask. The solution was sonicated for 5 min to ensure complete dissolution of the solid. Following sonication, the solution was allowed to equilibrate to room temperature after which it was diluted to volume with (50:50) vol%/vol% H_2O:CH_3CN. Working standards of **I** were prepared from the 500 μg/mL stock solution at 100, 70, 40, 20, 10, 2, 1, 0.5, and 0.2 μg/mL using (50:50) vol%/vol% H_2O:acetonitrile as the diluent.

Solutions of **I** stored at ambient laboratory conditions were demonstrated to be stable for at least 21 weeks.

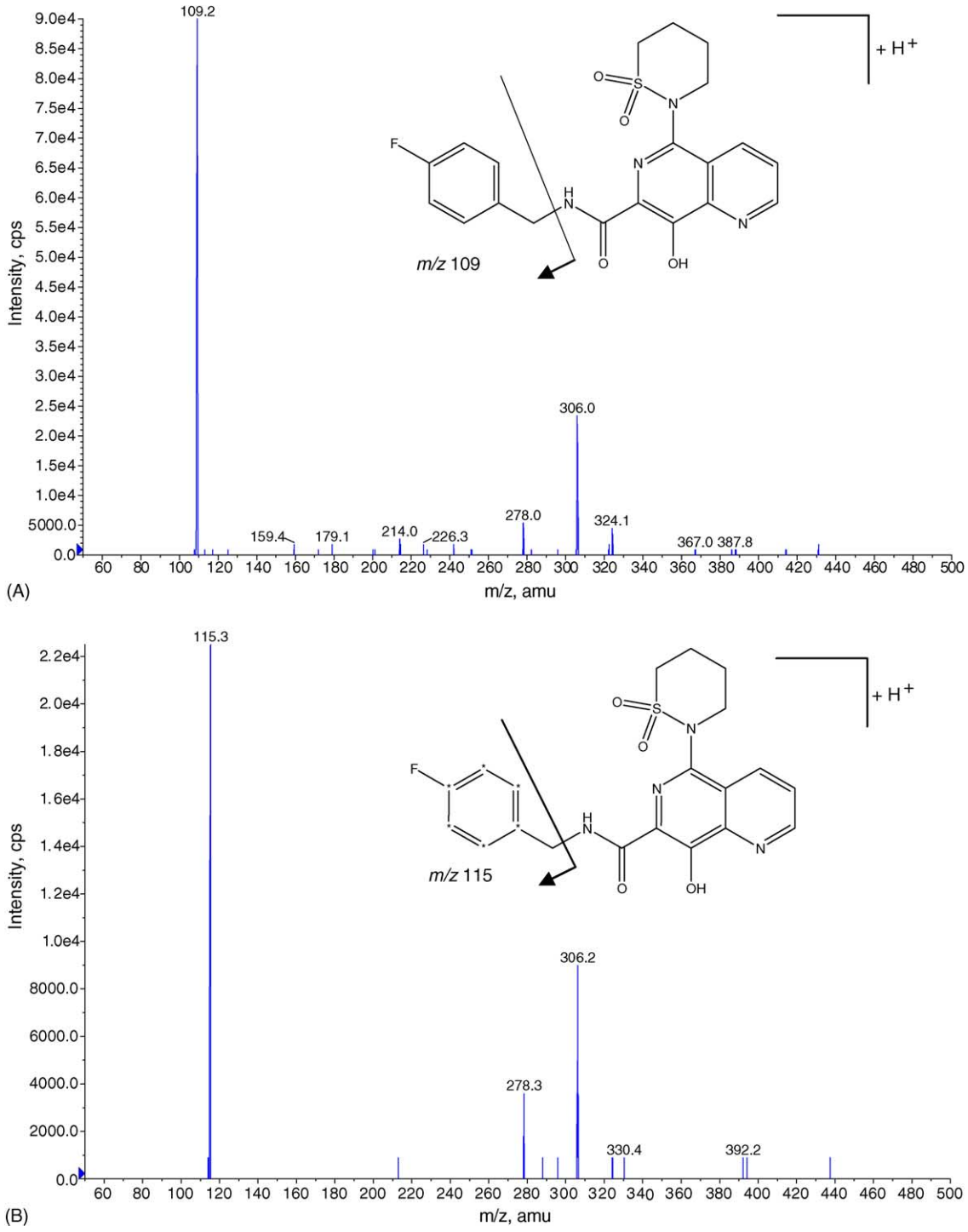

Fig. 2. Product ion mass spectra: (A) **I**; (B) **II**.

Plasma standards were prepared by spiking 25 μL of each working standard into 0.5 mL of human control plasma. These standards were used to construct calibration curves for the quantitation of **I** at plasma concentrations ranging from 10 to 5000 ng/mL. Samples found to contain **I** at concentrations above 5000 ng/mL were diluted appropriately with control plasma and re-assayed.

2.6. Sample preparation

Plasma samples were removed from −20 °C storage and immersed in a heated (37 °C) water bath for 30 min to thaw. After vortexing and centrifugation (2560 × *g* for 5 min) of the sample tubes, a 0.5 mL aliquot of plasma was transferred to a 7 mL polypropylene tube. A 25 μL aliquot of

(50:50) vol%/vol% H_2O:CH_3CN was added to the blanks, quality controls, and subject samples to compensate for the volume of diluent added during spiking of the calibration standards. Next, 25 μL of the working internal standard solution (2 μg/mL **II** in (50:50) vol%/vol% H_2O:acetonitrile) was added followed by 25 μL of 85% (w/v) H_3PO_4. After vortex mixing for 30 s, the sample was diluted with 0.5 mL H_2O.

Ansys SPEC C-8 96-well solid phase extraction plates (Varian, Inc., Walnut Creek, CA, USA) were conditioned with 0.5 mL methanol followed by 1 mL H_2O. The entire volume of the acidified and diluted plasma sample was applied to the plate using an 8-channel pipet and drawn through the wells by vacuum. The sorbent was washed with 1 mL H_2O followed by 1 mL H_2O:methanol (65:35) vol%/vol%. Next, the extraction plate was placed on top of a polystyrene ELISA plate (Fisher Scientific, Pittsburgh, PA, USA) and centrifuged for 3 min at $1006 \times g$ to draw the residual wash solvent through the wells. To elute the analytes, 200 μL of (20:80) H_2O:acetonitrile was passed though the wells by centrifugation for 5 min at $644 \times g$ and collected into a polypropylene collection plate (Microliter Analytical Supplies, Suwanee, GA, USA). To ensure compatibility with the HPLC mobile phase, the extracts were diluted with 200 μL of 25 mM ammonium formate pH 3.0 and vortex mixed. Finally, the diluted extracts were passed through a 96-well 0.45 μm nylon filter plate (Applied Separations, Allentown, PA, USA) into a clean polypropylene collection plate and transferred to the HPLC–MS/MS system for analysis.

3. Results and discussion

3.1. Optimization of the extraction procedure

Initial experiments performed using the Ansys SPEC C-8 plate, in which plasma was diluted with an equal volume of H_2O prior to extraction, yielded low and variable recovery of **I** (approximately 35%). The source of the low extraction recovery was traced to breakthrough of **I** during the sample loading step. In that **I** is weakly acidic (pK_a of the naphthyridine −OH is 7.3), plasma samples were acidified with 0.1N HCl or 0.1 M phosphate buffer (pH 3) prior to extraction in order to ensure protonation of **I** in an effort to more effectively retain the compound on the sorbent. However, neither of these conditions affected analyte recovery. In light of these results and the fact that **I** was known to be highly bound to human plasma proteins (unbound fraction <2%), it was hypothesized that protein binding of **I** was contributing to the low extraction recovery. Experience has shown that the addition of 25–50 μL of concentrated H_3PO_4 per mL of plasma is often effective at improving the extraction recovery of highly protein bound analytes. In the present case, the addition of 25 μL of concentrated (85%) H_3PO_4 to 0.5 mL plasma followed by dilution of the acidified sample with 0.5 mL H_2O was found to improve the absolute recovery of **I** (approximately 70%) as well as decrease the variability.

Optimization of the elution solvent was next investigated. Elution solvents consisting of various proportions of water and acetonitrile were evaluated. It was found that elution with 200 μL of (20:80) vol%/vol% H_2O:acetonitrile yielded recovery comparable to that obtained eluting with the same volume of neat acetonitrile. As the former was a considerably weaker elution solvent, it was employed to afford more selective elution of the analytes. Drawing of the elution solvent through the wells by centrifugation rather than vacuum was found to provide more consistent recovery, presumably due to a more uniform flow rate.

3.2. Optimization of analyte recovery from frozen plasma

Poor recovery of **I** from plasma samples that had been spiked prior to freezing was observed during the initial analysis of quality control (QC) samples (Table 1). Given that the recovery of **I** from the low and mid quality control samples did not appear to be compromised (mean accuracies 107 and 101%, respectively) on freezing and thawing, the poor recov-

Table 1
Effect of sample heating on the recovery of **I** from human plasma following freezing and thawing[a]

	Assayed concentration (ng/mL)					
	Thawed at 37 °C for 30 min			Thawed unassisted at room temperature		
	30 QC	500 QC	3000 QC	30 QC	500 QC	3000 QC
	31.7	507.0	2800.2	33.5	514.3	2342.3
	31.8	506.7	2767.4	32.0	499.4	2319.3
	32.0	522.1	2856.4	32.4	505.6	2274.0
	32.6	511.7	2963.4	31.2	505.9	2261.2
	32.5	501.1	2898.1	31.4	498.8	2454.1
Mean	32.1	509.7	2857.1	32.1	504.8	2330.2
C.V.	1.3	1.5	2.7	2.9	1.2	3.3
Accuracy[b] (%)	107.1	101.9	95.2	107.0	101.0	77.7

[a] Plasma samples were spiked with **I** and stored at −20 °C for at least 12 h prior to thawing and processing.
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

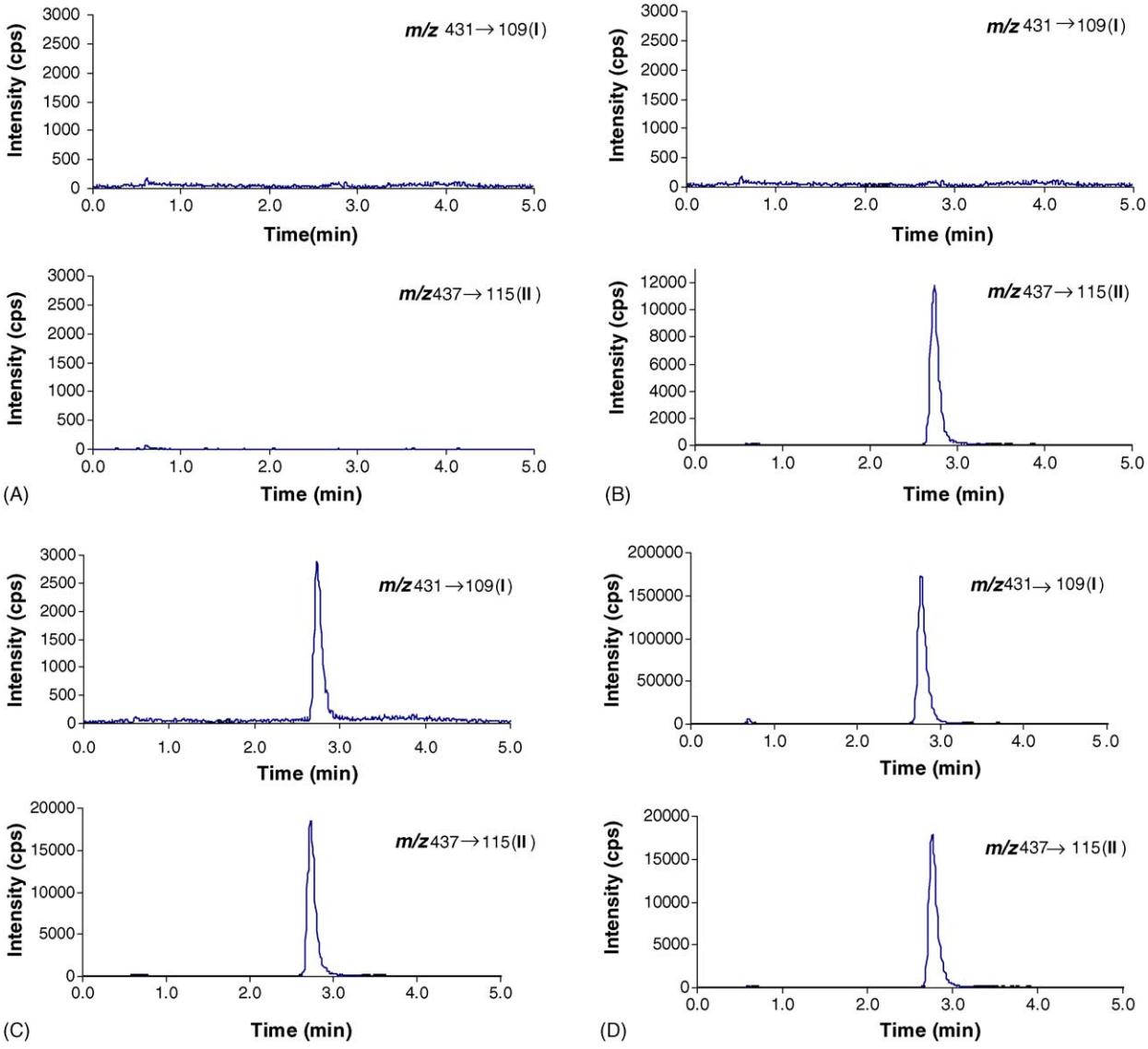

Fig. 3. Representative chromatograms: (A) control plasma double blank; (B) control plasma single blank (plasma concentration of **II** was 100 ng/mL); (C) 10 ng/mL plasma standard; (D) plasma sample collected from a subject 5 h after receiving a 200 mg oral dose of **I**. The assayed concentration of **I** in this sample was 665.0 ng/mL.

ery of **I** for the high QC was attributed to the low aqueous solubility of the compound.

In an effort to improve the recovery of **I** from the high QC samples, mild heating of the samples was investigated. A set of QC samples ($n = 5$ at each concentration) was removed from −20 °C storage and immersed in a 37 °C water bath for 30 min to thaw. A second set of QC samples was thawed unassisted at room temperature to serve as a control group. As shown in Table 1, the mean accuracy of the 3000 ng/mL QC improved from 77.7% in the control to 95.2% when the samples were thawed in the heated bath. No significant difference was observed between the two groups of low or mid QCs. Further experiments (detailed below) confirmed the stability of **I** in plasma following repeated freeze–thaw cycles performed under these conditions.

3.3. Method validation

The method was validated according to FDA guidelines [6]. The validation experiments and results obtained are described below.

3.3.1. Selectivity

Assay selectivity was evaluated by analyzing six separate lots of drug free human control plasma. All plasma lots were found to be free of interferences with the compounds of interest.

In addition, the HPLC–MS/MS system was evaluated for the presence of "cross-talk" between the channels used for monitoring **I** and **II**. Plasma samples spiked with the working concentration of **II** (100 ng/mL) in the absence of **I** (i.e. control plasma single blanks) were prepared and analyzed. No

peaks were detected in the channel used to monitor **I** (Fig. 3). Additionally, a plasma sample spiked with **I** at the assay upper limit of quantitation in the absence of **II** was analyzed. No "cross-talk" was observed.

Representative chromatograms of a control plasma double blank, control plasma single blank, 10 ng/mL plasma standard, are shown in Fig. 3A–C.

3.3.2. Sensitivity and linearity

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 15% of nominal and precision not exceeding 15% C.V., was 10 ng/mL.

Calibration curves were constructed by plotting the peak area ratios (**I**/**II**) of plasma standards versus nominal concentration. Weighted ($1/x^2$, where x = nominal standard concentration) linear least squares regression was employed. Linear calibration curves were obtained over the range 10–5000 ng/mL **I** in plasma. Unknown sample concentrations exceeding 5000 ng/mL were diluted appropriately with control plasma and re-assayed.

3.3.3. Accuracy and precision

The within-day accuracy and precision of the assay were determined by analyzing replicate ($n = 5$) standard curves. To more fully characterize the ruggedness of the assay, the standard curves were prepared in five different lots of plasma (i.e. lots originating from five unique donors). The results of this analysis are provided in Table 2. The ruggedness of the assay and the absence of relative matrix effects are further evidenced by the precision of the slopes of the individual standard curves, which is shown in Table 3.

Quality control (QC) samples were prepared at low (30 ng/mL), medium (500 ng/mL), and high (3000 ng/mL) concentrations and stored under the same conditions as the clinical samples (i.e. −20 °C) for the purpose of evaluating sample stability and inter-day accuracy and precision. Duplicate QC samples at each concentration were analyzed daily along with standards and clinical samples. Inter-day accuracy and precision data for QC samples analyzed over a 3-month period are provided in Table 4.

Table 2
Intra-day accuracy and precision for the determination of **I** in five unique lots of human plasma

Nominal concentration (ng/mL)	Mean determined concentration (ng/mL, $n = 5$)	Accuracy[a] (%)	Precision[b] (%)
10	10.2	102.0	3.0
25	24.1	96.6	2.4
50	49.0	98.1	0.7
100	97.3	97.3	2.5
500	503.1	100.6	1.9
1000	1036.4	103.6	2.3
2000	2039.7	102.0	3.6
3500	3514.7	100.4	3.1
5000	4973.3	99.5	1.5

[a] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Precision is expressed as the coefficient of variation of peak area ratios.

Table 3
Intra-day slope precision for standard curves prepared in five unique lots of human plasma

Control plasma lot	Slope
1	0.01601
2	0.01537
3	0.01555
4	0.01542
5	0.01578
Mean	0.01562
C.V. (%)	1.7

3.3.4. Extraction recovery

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) was prepared by spiking **I** into plasma at 50, 500, and 3500 ng/mL. Each of the samples was also spiked with **II** at the working concentration of 100 ng/mL. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of **I** and **II** that were added

Table 4
Inter-day QC accuracy and precision

Run[a]	Assayed concentration (ng/mL)[b]		
	Low QC	Mid QC	High QC
1	31.0	497.0	2756.8
2	31.0	515.5	2807.5
3	31.0	508.4	2826.1
4	30.5	495.4	2761.6
5	32.0	512.1	2930.0
6	32.6	534.4	2916.3
7	33.4	530.4	2966.0
8	32.0	528.5	2875.9
9	30.4	495.2	2903.2
10	31.2	509.0	2883.5
11	32.0	517.1	2942.5
12	32.2	512.9	2966.2
13	32.9	512.0	2950.2
14	33.1	520.1	2915.5
15	31.4	494.5	2805.7
16	31.1	516.3	2885.8
17	31.4	506.0	2894.8
18	33.6	511.9	2872.7
19	31.1	501.9	2818.3
20	32.3	513.3	2937.6
21	29.1	485.0	2688.7
22	31.8	523.5	2916.2
Mean	31.7	510.9	2873.7
Accuracy[c] (%)	105.5	102.2	95.8
C.V. (%)	3.4	2.5	2.6

[a] Runs were performed over a 3-month period.
[b] Data presented are the mean of duplicate QC samples at each concentration.
[c] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 5
Benchtop stability of **I** in human plasma

Nominal concentration (ng/mL)	$t = 0$ h (control) mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	$t = 4$ h mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	Difference from control (%)
30	31.3 (2.5)	104.2	32.1 (2.5)	107.0	2.7
500	490.1 (1.9)	98.0	503.6 (2.8)	100.7	2.8
3000	2767.5 (3.8)	92.3	2826.8 (2.6)	94.2	2.1

[a] $n = 3$; numbers in parentheses are coefficients of variation (C.V.).
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

to the pre-extraction spiked samples. Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to the raw peak areas of the samples spiked after extraction. Mean extraction recovery for **I** and **II** was 69%.

The extent of matrix suppression or enhancement of ionization was assessed by comparing the mean raw peak areas of the post-extraction spiked plasma samples to the mean raw peak areas of **I** and **II** in neat solution at the appropriate concentrations. Although some matrix enhancement of ionization was observed for **I** and **II** ($\text{area}_{\text{post-ext}}/\text{area}_{\text{neat}} = 1.12$ for **I** and 1.13 for **II**), assay precision was not compromised by a relative matrix effect, as illustrated in Tables 1 and 2.

3.3.5. Benchtop stability

The stability of **I** in plasma exposed to ambient laboratory conditions prior to processing was investigated. Quality control samples ($n = 3$ at three concentration levels) were removed from −20 °C storage, thawed by immersion in a 37 °C water bath, and allowed to sit on a laboratory bench top for 4 h prior to processing. A set of QC samples ($n = 3$ per concentration level) processed immediately after thawing served as a control group. The results, which are presented in Table 5, demonstrate the stability of **I** under these conditions.

3.3.6. Freeze–thaw stability/stability under HIV deactivation conditions

The stability of **I** in human plasma over multiple freeze–thaw cycles was assessed. QC samples were subjected to 3 freeze–thaw cycles and analyzed along with a set of control QCs subjected to a single freeze–thaw cycle. Each freeze–thaw cycle consisted of storage at −20 °C for a minimum of 12 h followed by thawing by immersion in a 37 °C water bath for 30 min. As shown in Table 6, **I** was found to be stable through 3 cycles of freezing and thawing.

In that the assay would ultimately be used to analyze samples collected from HIV positive patients, the stability of **I** in human plasma subjected to heating at 56 °C for 120 min was assessed. Heating of plasma samples in this manner is used to deactivate the human immunodeficiency virus, significantly reducing the possibility of infection of laboratory personnel during sample handling and processing. Results obtained for plasma samples spiked with **I** and heated under the conditions described above showed no significant difference from a control group of samples prepared similarly but not subjected to heat treatment (data not shown).

3.3.7. Processed sample stability/re-injection accuracy and precision

The stability of **I** in processed samples was investigated as follows. A set of samples consisting of a standard curve and QCs ($n = 5$ per concentration) was processed and analyzed. After this analysis, the samples were allowed to remain on the autosampler exposed to ambient laboratory conditions for 6 days, after which the samples were re-injected. Assayed QC concentrations were determined based on the initial injection of the standard curve. The results, which are presented in Table 7, demonstrate the stability of **I** in processed samples.

Sample re-injection accuracy and precision was assessed in the same experiment. Assayed concentrations of the re-injected QC samples were determined using the re-injected standard curve and compared with the assayed concentrations determined from the original injection of the samples. As indicated in Table 7, no meaningful difference in accuracy or precision was found among these data sets.

3.3.8. Sample dilution

To demonstrate the ability to dilute and analyze samples containing **I** at concentrations above the assay upper limit of quantitation, a set of plasma samples was prepared containing **I** at a concentration of 12.5 μg/mL and placed in a −20 °C freezer overnight prior to analysis. After thawing by immersion in a 37 °C water bath, a 100 μL aliquot was withdrawn for analysis ($n = 5$), diluted with 400 μL of control

Table 6
Freeze–thaw stability of **I** in human plasma

Nominal concentration (ng/mL)	1 F/T cycle (control) mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	3 F/T cycles mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	Difference from control (%)
30	30.6 (2.1)	102.0	31.0 (2.1)	103.4	1.4
500	503.4 (1.7)	100.7	511.4 (0.8)	102.3	1.6
3000	2768.9 (1.5)	92.3	2781.2 (1.4)	92.7	0.4

[a] $n = 3$; numbers in parentheses are coefficients of variation (C.V.).
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 7
Processed sample stability and re-injection accuracy and precision

Nominal concentration (ng/mL)	Initial injection[a]		Re-injection[b]		Re-injection[c]	
	Mean assayed concentration (ng/mL)[d]	Accuracy[e] (%)	Mean assayed concentration (ng/mL)[d]	Accuracy[e] (%)	Mean assayed concentration (ng/mL)[d]	Accuracy[e] (%)
30	30.8 (2.1)	102.7	30.6 (4.4)	102.1	30.5 (4.6)	101.8
500	507.4 (1.5)	101.5	504.6 (2.5)	100.9	510.5 (2.5)	102.1
3000	2775.1 (1.3)	92.5	2769.5 (2.1)	92.3	2802.7 (2.1)	93.4

[a] Initial injection of QCs calculated from initial injection of standard curve.
[b] Re-injected QCs calculated from re-injected standard curve.
[c] Re-injected QCs calculated from initial injection of standard curve.
[d] $n = 5$; numbers in parentheses are coefficients of variation (% C.V.).
[e] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 8
Sample dilution accuracy and precision

Assayed concentration (ng/mL)	Reported concentration (ng/mL)
2310.9	11554.5
2290.7	11453.5
2354.5	11772.5
2284.6	11423.0
2320.6	11603.0
Mean	11599.5
C.V.	1.5
Accuracy (%)	92.8

Nominal concentration = 12,500 ng/mL; dilution factor = 5.

human plasma, and processed as described in Section 2.6. The results of this experiment are shown in Table 8.

3.4. Application of the assay

The assay has successfully been utilized to analyze samples obtained from subjects administered oral doses of **I**. A representative chromatogram from a post-dose sample is provided in Fig. 3D. Following the administration of 200 mg of the compound, plasma concentrations of **I** ranged from 21.1 to 1500 ng/mL. The method was found to be suitable for determining the plasma concentrations of **I** for up to 12 h following a 200 mg dose.

4. Conclusion

An HPLC–MS/MS assay has been developed and validated for the determination of **I** in human plasma. This assay was demonstrated to be selective, accurate, and precise and was suitable for the analysis of samples collected during clinical studies of this compound.

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