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Determination of the HIV integrase inhibitor, MK-0518 (raltegravir), in human plasma using 96-well liquid–liquid extraction and HPLC-MS/MS

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Abstract

An HPLC-MS/MS method was developed for the determination of MK-0518 (raltegravir), an HIV integrase inhibitor, in human plasma over the concentration range of 2–1000 ng/mL. Stable isotope labeled $^{13}C_6$-MK-0518 was used as an internal standard. The sample preparation procedure utilized liquid–liquid extraction with hexane:methylene chloride in the 96-well format with a 200 μL plasma sample size. The compounds were chromatographed on an Ace C_{18} (50 × 3.0 mm, 3 μm, titanium frits) column with 42.5/57.5 (v/v %) 0.1 mM EDTA in 0.1% formic acid/methanol mobile phase at a flow rate of 0.5 mL/min. Multiple reaction monitoring of the precursor-to-product ion pairs for MK-0518 (*m/z* 445 → 109) and $^{13}C_6$-MK-0518 (*m/z* 451 → 367) on an Applied Biosystem API 4000 HPLC-MS/MS was used for quantitation. Intraday precision of standard curve concentrations in five different lots of control plasma was within 3.2%, while accuracy ranged from 94.8 to 106.8%. The mean extraction recovery of spiked plasma samples was 87%. Quality control (QC) samples were stored at −20 °C. Initial within day analysis showed QC accuracy within 7.5% of nominal with precision of 3.1% or less. The plasma QC samples were demonstrated to be stable for up to 23 months at −20 °C. The method described has been used to support over 18 clinical studies during Phase I through III of clinical development.

Keywords: Integrase inhibitor; Raltegravir; MK-0518; Plasma; Bioanalytical; HPLC–MS/MS; Liquid–liquid extraction

1. Introduction

Studies have indicated that nearly 80% of HIV positive patients with detectable viral loads are harboring a virus that is resistant to at least one of the more than 20 currently marketed anti-retrovirals (ARV) [1]. It has also been shown that up to 24% of newly infected, treatment-naïve patients are carrying a resistant virus [2]. In addition, most ARVs from the same class have similar chemical structures so resistance to one drug can lead to cross-resistance to other drugs within that class [3]. Consequently, novel drugs working through different mechanisms of action are needed to counteract resistance and compliment existing treatment options.

MK-0518, also known as raltegravir, is in Phase III clinical development for the treatment of human immunodeficiency virus (HIV) infection. MK-0518 belongs to a new class of antiretroviral agents called integrase inhibitors. Integrase inhibitors work by inhibiting the insertion of viral DNA into the genome of the cell, thus preventing replication of the virus [4]. MK-0518 has proven to be effective at reducing the viral RNA load of treatment-naïve HIV patients and patients with multidrug-resistant strains of the virus during clinical studies [5,6].

A bioanalytical method to determine the concentration of MK-0518 in human plasma was required to support clinical development studies. The development and validation of a

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method utilizing liquid–liquid extraction in the 96-well format for sample preparation and HPLC with tandem mass spectrometric detection is described in this paper.

2. Experimental

2.1. Materials

MK-0518 (Fig. 1) was obtained from the Chemical Data Department at Merck Research Laboratories (Rahway, NJ, USA) as its potassium salt. Stable isotope labeled $^{13}C_6$-MK-0518 (Fig. 1) was provided by Labeled Compound Synthesis, Drug Metabolism Department (Rahway, NJ, USA). Methanol, hexane, methylene chloride (Optima Grade), and acetic acid (99.4%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium acetate (99.9%), EDTA (99.999%), and formic acid (95%) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). K_2EDTA human control plasma was purchased from Biological Specialty Corporation (Colmar, PA, USA). A Millipore Milli-Q Plus (Bedford, MA USA) was used to generate deionized (18 mΩ/cm) water in-house.

2.2. Instrumentation

The HPLC-MS/MS system consisted of a Varian ProStar 430 autosampler (Walnut Creek, CA USA), Perkin Elmer series 200 micropumps (Norwalk, CT, USA), and an Applied Biosystem API 4000 (Foster City, CA, USA) triple quadropole mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface. Analyst version 1.1 software was used for data acquisition and processing. A 96-well liquid–liquid extraction procedure was accomplished by the use of a Tomtec Quadra 96, model 320 (Hamden, CT, USA).

2.3. Chromatographic conditions

Chromatography was performed on a 3.0 × 50 mm Ace C_{18} (Mac-Mod, Chadds Ford, PA, USA) column (3 μm, titanium frits) with mobile phase consisting of 42.5/57.5 (v/v %) 0.1 mM EDTA in 0.1% formic acid/methanol at a flow rate of 0.5 mL/min. A 5 μL full loop injection was used with a total run time of 3.5 min.

2.4. Mass spectrometer conditions

An atmospheric pressure chemical ionization interface (APCI) probe in the positive mode set at 600 °C was used to ionize the sample. Collision induced dissociation of the protonated molecules was achieved with a CAD gas setting of 7, the corona discharge needle voltage was optimized at 4, and the dwell time for analyte and ISTD was 350 ms.

2.5. Preparation of standards

A 500 μg/mL stock solution of MK-0518 was prepared and serially diluted to give working standard solutions of 0.02, 0.05, 0.1, 0.5, 1, 5, 7.5, and 10 μg/mL. A 100 μg/mL stock solution of stable isotope labeled internal standard ($^{13}C_6$-MK-0518) was prepared and diluted to a 1 μg/mL working solution. All solutions were prepared in 50/50 (v/v%) acetonitrile/water. Solutions were stored at ambient conditions in amber glass.

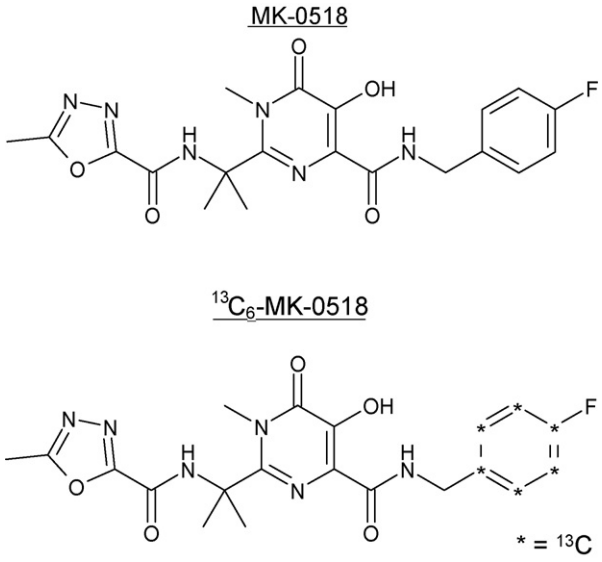

Fig. 1. Chemical structures of MK-0518 and $^{13}C_6$-MK-0518 (ISTD).

Plasma standards were prepared by spiking 20 μL of working standard and 20 μL of working ISTD into 200 μL of K_2EDTA human control plasma. These standards were used to quantitate clinical plasma samples over the concentration range of 2–1000 ng/mL. Clinical samples that contained more than 1000 ng/mL MK-0518 were diluted with control plasma and reanalyzed.

2.6. Preparation of quality control samples

Low, middle, and high quality control stock solutions at 0.6, 7.5, and 75 μg/mL were prepared by diluting a separate 500 μg/mL MK-0518 solution with 50/50 (v/v%) acetonitrile/water. Plasma QC samples were prepared by transferring 0.5 mL of these stock solutions into a 50-mL volumetric flask and filling the flask to the mark with human control plasma. Eight hundred microliter aliquots were transferred to 3.6 mL cryo tubes and stored at −20 °C.

2.7. Sample preparation

Two hundred microliter aliquots of plasma samples were pipetted into a 2.2 mL polypropylene 96-square well plate (Arctic White, Bethlehem, PA, USA) and spiked with 20 μL of a 1 μg/mL working internal standard solution and 20 μL of (50:50) v/v% ACN:H_2O to compensate for the volume of working standard solution used to prepare standard curve samples. Using a Tomtec Quadra 96, 150 μL of a 0.2 M ammonium acetate pH 4 solution and 1.2 mL (50:50) v/v% hexane:methylene chloride extraction solvent was added to each well. The plate was sealed with a 96-well square silicone/PTFE mat (Arctic White, Bethlehem, PA, USA) and rotate mixed for

20 min. The extraction plate was then centrifuged for 5 min and using the Tomtec Quadra 96, 825 μL of the organic layer was transferred to a clean 1.2 mL 96-well polypropylene autosampler plate (Arctic White, Bethlehem, PA, USA). The solvent was evaporated to dryness under N_2 at 40 °C for approximately 10 min. The samples were then reconstituted in 350 μL 45/55 (v/v %) 0.1 mM EDTA in 0.1% formic acid/methanol reconstitution solvent, capped with a polyethylene sealing mat (Arctic White, Bethlehem, PA, USA), mixed, and 5 μL was injected onto the HPLC–MS/MS system for analysis.

3. Results and discussion

3.1. Assay development

3.1.1. Analyte detection

Precursor ions for MK-0518 and internal standard were determined from Q1 spectra obtained during the infusion of neat solutions in mobile phase, via the APCI source, into the mass spectrometer operating in the positive ionization mode with the collision gas off. Under these conditions, the protonated molecules at *m/z* 445 and 451 for MK-0518 and internal standard, respectively, were predominately observed. Each of the precursor ions was subjected to collision induced dissociation (CID) in order to determine the resulting product ions. Product ion mass spectra for MK-0518 and internal standard are shown in Fig. 2. Initially, multiple reaction monitoring of the predominant precursor to product ion pairs *m/z* 445 → 109 for MK-0518 and *m/z* 451 → 115 for ISTD was used for quantitation. A high background noise level, however, was observed for the *m/z* 451 → 115 ISTD channel, possibly due to a component of the mobile phase. Thus, an alternative transition, *m/z* 451 → 367, which did not have a raised baseline, was employed for the ISTD.

3.1.2. Chromatographic system

An ACE C_{18} column was successfully used for the analysis of a previous integrase inhibitor within our laboratory;

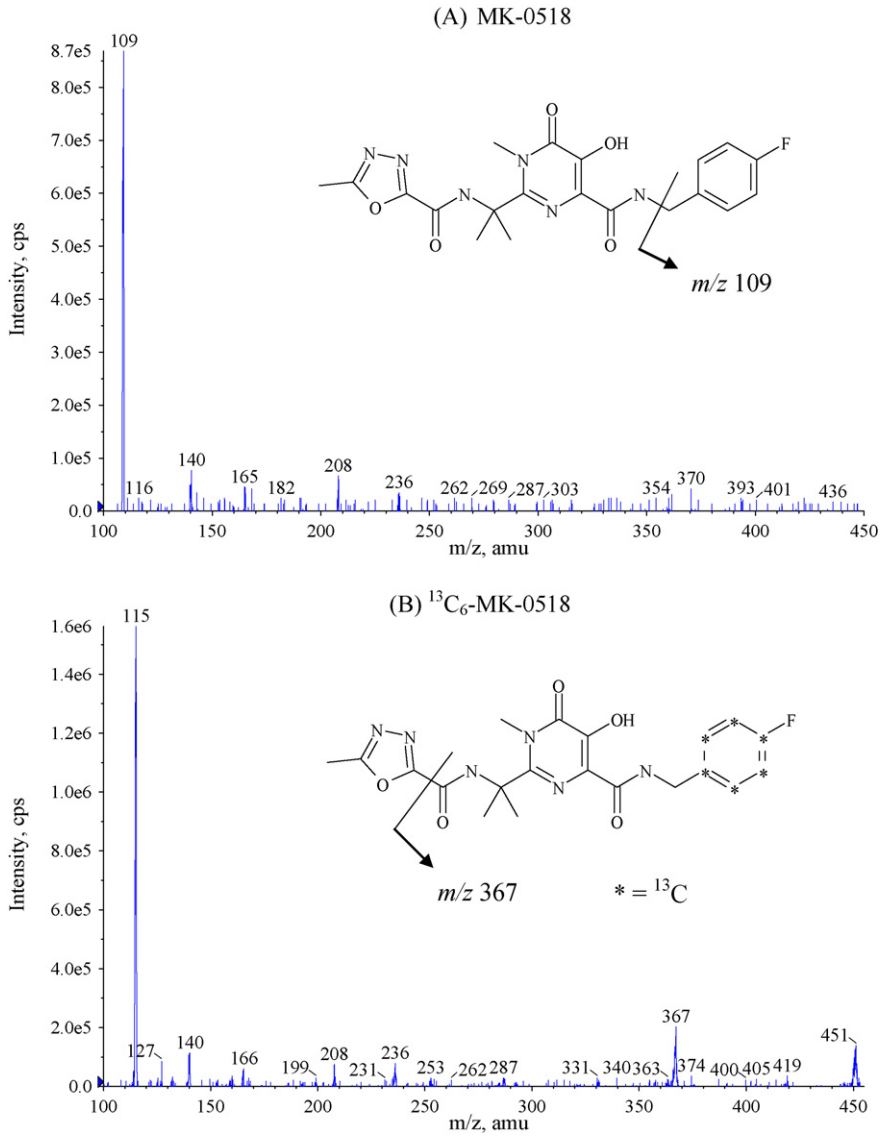

Fig. 2. Product ion mass spectrum of MK-0518 and $^{13}C_6$-MK-0518.

hence this column was evaluated and found to provide adequate peak shape and resolution for the analysis of MK-0518. The low concentration of EDTA (0.1 mM in free acid form) was added to the mobile phase for a previous compound in this structural class in order to counteract known chelating of the compound to metals within the HPLC-MS/MS system (unpublished work). The mobile phase was kept consistent for this compound due to its similar structure. The EDTA did not have a negative impact on the operation of the API 4000 mass spectrometer or on the ionization sensitivity of the compound.

3.1.3. Extraction method

Due to the fact that antiretroviral plasma concentrations are frequently monitored, we desired to develop a simple and inexpensive extraction method that could be readily implemented in monitoring labs. Therefore, our work focused on the use of liquid–liquid extraction. Hexane:methylene chloride, MTBE, and ethyl acetate were evaluated as extraction solvents under pH 2–9 conditions. The best recovery was observed with the plasma buffered to pH 4 using 1:1 hexane:methylene chloride as the extraction solvent.

3.2. Assay validation

Assay validation was performed in accordance with current guidelines [7,8].

3.2.1. Representative chromatograms

Representative chromatograms, extracted and analyzed under the conditions of the assay, of a control double blank sample, a control single blank sample containing 100 ng/mL of ISTD, and a 2 ng/mL MK-0518 plasma standard (LLOQ) are shown in Fig. 3a–c.

3.2.2. Intraday variability

An assessment of intraday variability was conducted by analyzing standard curve samples in five different lots

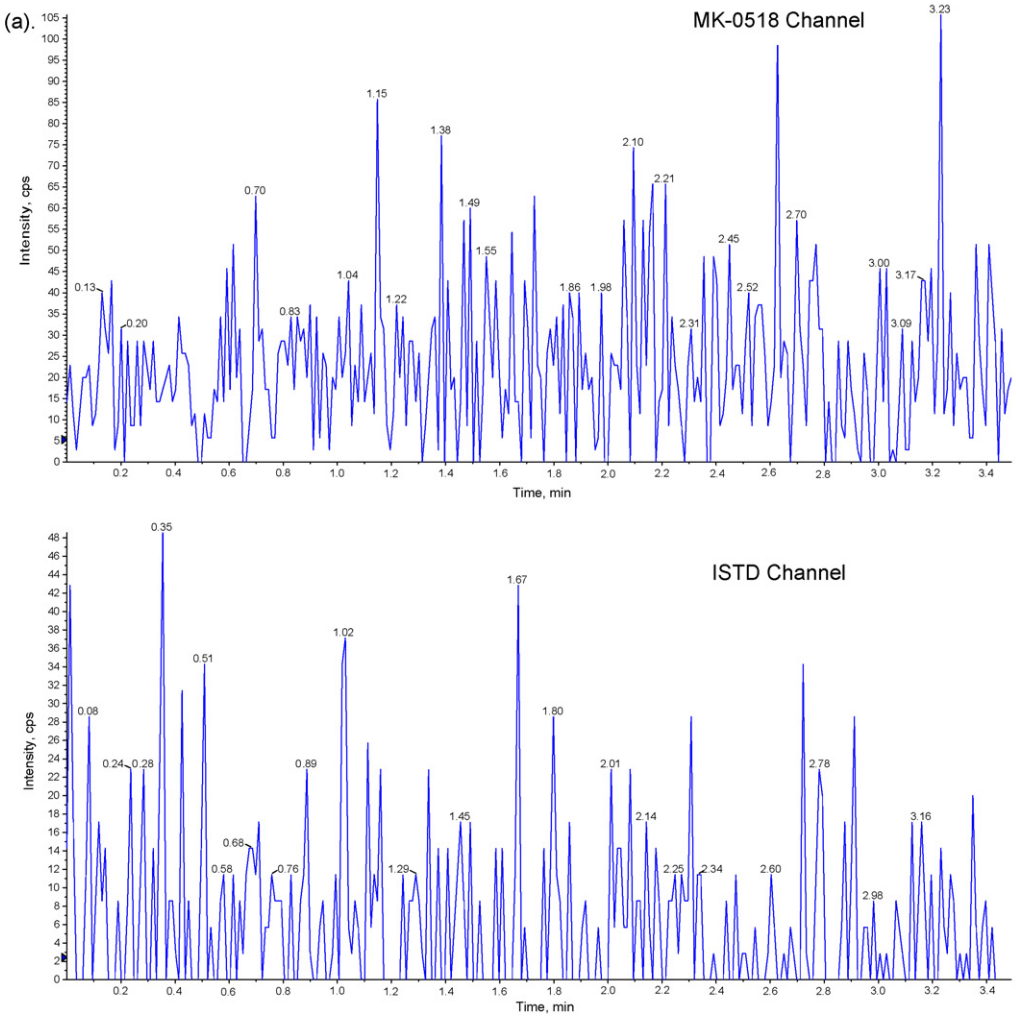

Fig. 3. (a) Representative chromatogram of a control plasma double blank sample. (b) Representative chromatogram of a control plasma single blank sample containing 100 ng/mL ISTD. (c) Representative chromatogram of an extracted 2 ng/mL MK-0518 standard in control plasma. (d) Representative chromatogram from a clinical subject collected 1 h after dosing with 400 mg MK-0518. This sample was extracted after a 10-fold dilution with human control plasma and the resulting concentration was 5513.6 ng/mL.

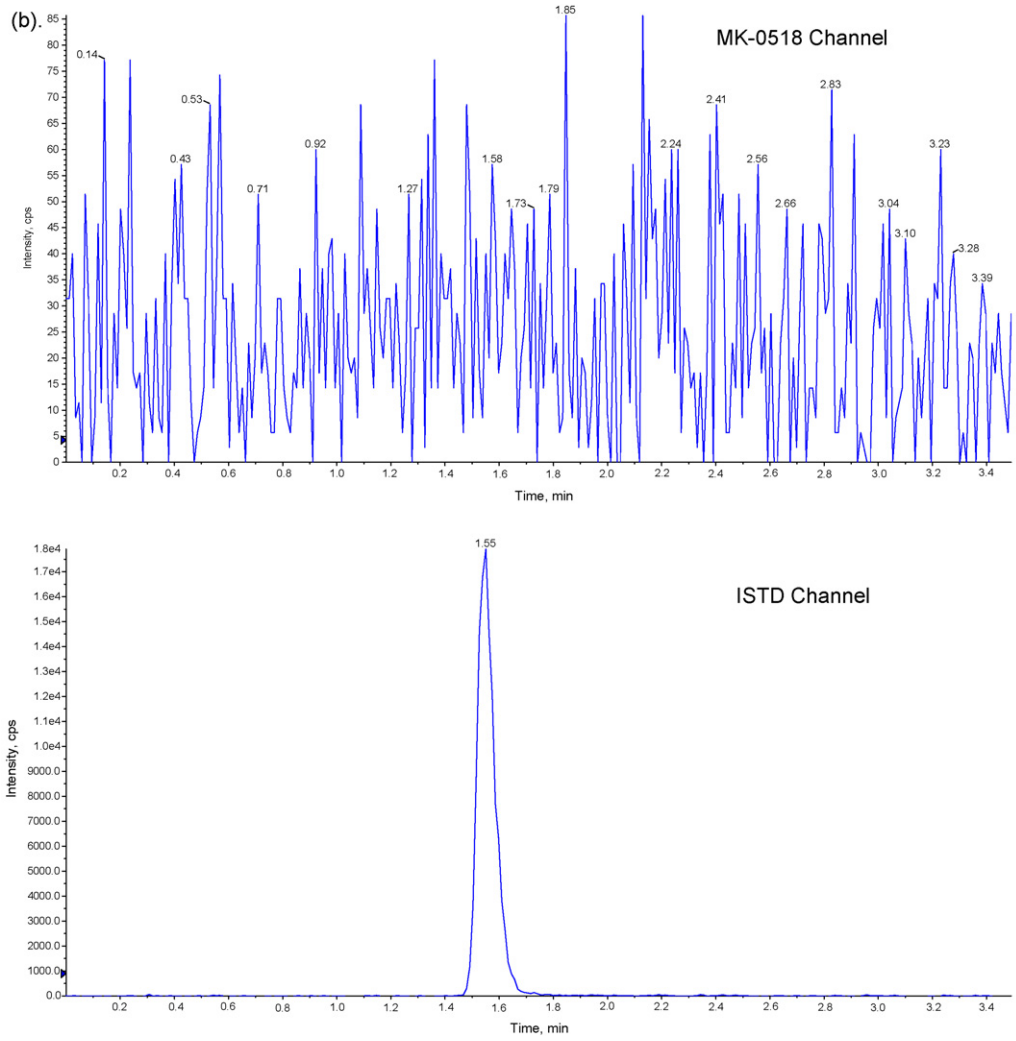

Fig. 3. (*Continued*)

of human control plasma over the calibration range of 2–1000 ng/mL. The resulting precision and accuracy data are presented in Table 1. The coefficient of variation of the assay was ≤3.2% at all concentrations within the standard curve range, while the assay accuracy was 94.8–106.8% of nominal.

3.2.3. Quality control samples

Quality control (QC) samples containing MK-0518 were prepared at concentrations of 6.0 ng/mL (low QC), 75 ng/mL (middle QC) and 750 ng/mL (high QC). Initial within-day analysis of the QC samples was within 7.5% of nominal with a C.V. of 3.1% or below (Table 2).

3.2.4. Inter-day variability of quality control samples

Ruggedness of the described method was demonstrated through inter-day ($n = 38$) analysis of QC samples over a 5-month study span. The average QC accuracy was within 5.2% of nominal and the precision was 4.3% or below.

3.2.5. Extraction recovery and assessment of the matrix effect on ionization

Extraction recovery and the effect of the sample matrix on ionization were evaluated for MK-0518 using standard samples prepared at analyte concentrations of 5, 100, and 750 ng/mL in five different lots of control plasma. Recovery of the extraction was determined by comparing the absolute peak areas of the standards in human plasma extracted as described, to control plasma extracted in the same manner and then spiked post extraction with the same concentration of the drug and internal standard. Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent. Results are shown in Table 3. Based on the intra-day precision results that were obtained using five different lots of control plasma, a relative matrix effect was not expected to affect the assay (Table 3). The lack of a relative matrix effect is further illustrated in Table 4, where an examination of the slopes of the standard curves prepared in five different lots of control plasma

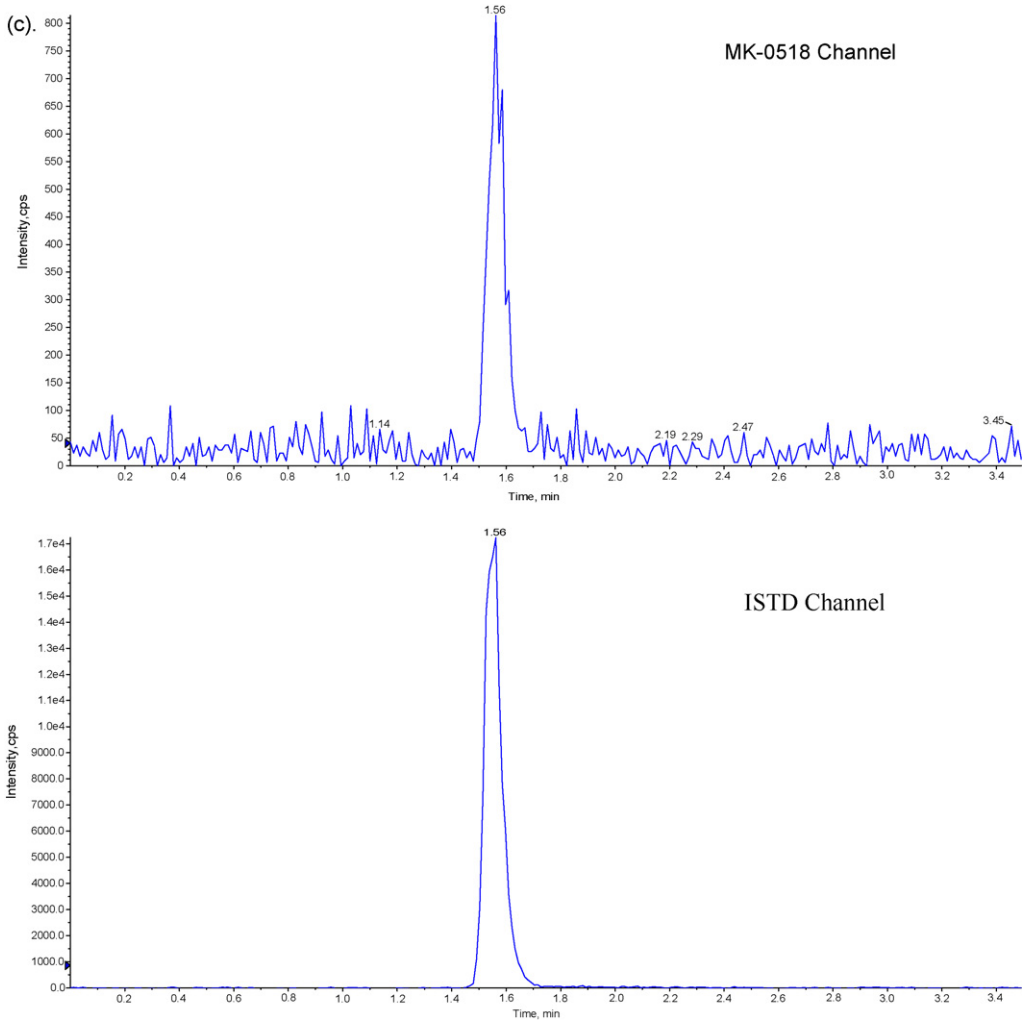

Fig. 3. (*Continued*)

shows a precision of 0.7%. Precision of standard curve slopes of less than 3–4% has been proposed to have no significant relative matrix effect [9].

3.2.6. Evaluation of "cross-talk"

The presence of "cross-talk" between channels used for monitoring the analytes was evaluated by the analysis of plasma samples containing $^{13}C_6$-MK-0518 at the working concentration (100 ng/mL) in the absence of MK-0518 and the analysis of plasma samples containing MK-0518 at the ULOQ (1000 ng/mL) in the absence of ISTD. No "cross-talk" was observed in either experiment.

3.2.7. Freeze–thaw stability

Quality control samples ($n = 5$ at each concentration) were subjected to freeze–thaw cycles consisting of a thaw to reach room temperature (≥4 h) and then refreezing (−20 °C) at least overnight and repeating for three cycles. Samples exposed to only one freeze–thaw cycle were used as the control samples. Samples that were subjected to additional freeze-thaw cycles analyzed within 1.6% of the controls. Complete results of the analysis are presented in Table 5.

3.2.8. Heat deactivation stability

HIV positive plasma samples are frequently heat deactivated prior to sample analysis to protect laboratory employees [10]. To determine the heat deactivation stability of MK-0518 in HIV positive plasma samples, QC tubes were submerged into a 56 °C water bath for 90 min. These samples were analyzed together with a control set of QCs. The results of the assessment are shown in Table 6. The mean concentration of the heat deactivated samples was within 6.7% of the control samples.

3.2.9. Room temperature stability

To assess room temperature stability of MK-0518 in human plasma, quality control samples were allowed to remain at room temperature for 5 h before analysis. These samples were analyzed and the results were compared with a set of samples that were assayed immediately after thawing. The results for the samples that were kept at room temperature prior to analysis were

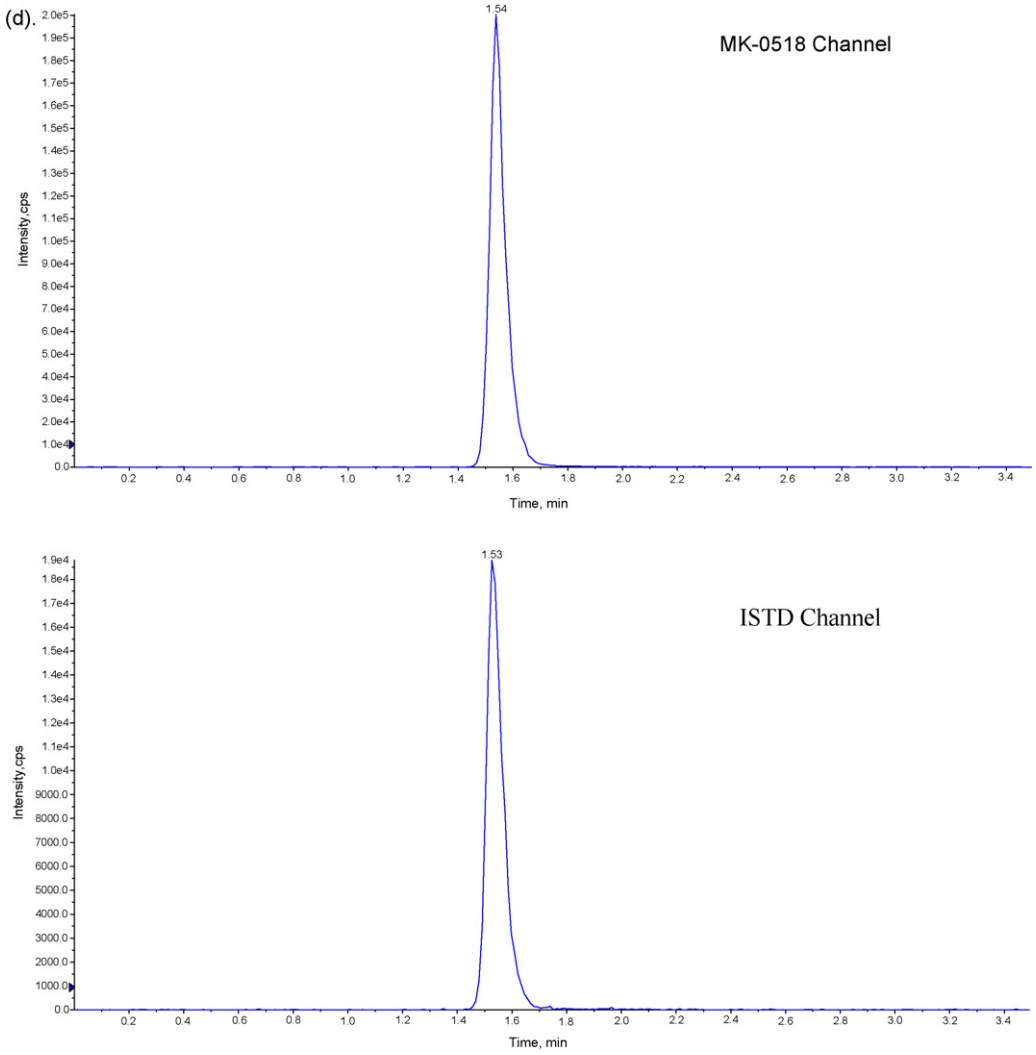

Fig. 3. (*Continued*)

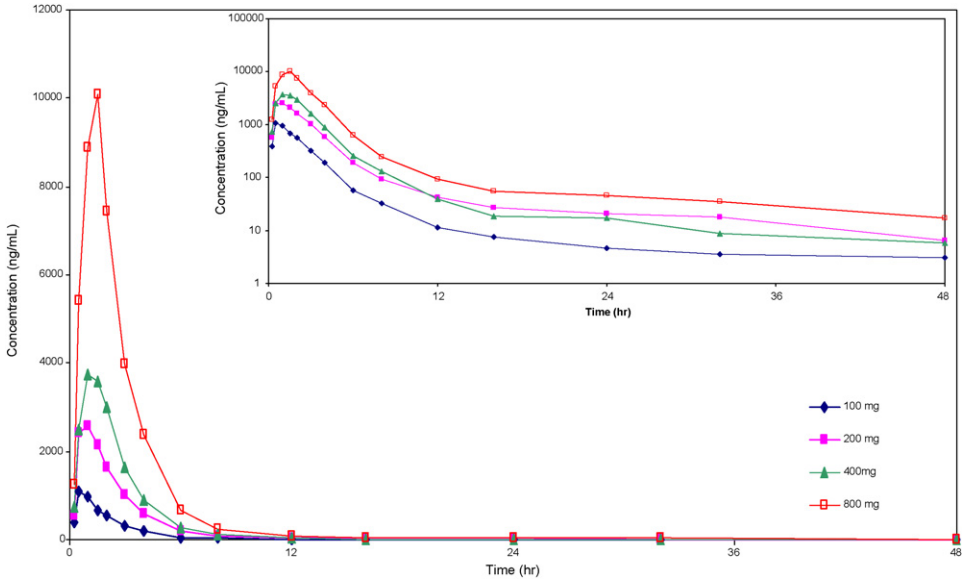

Fig. 4. Mean ($n = 6$) plasma concentration vs. time profile after a single 100, 200, 400, and 800 mg oral dose of MK-0518.

Table 1
Representative intraday precision and accuracy data for the determination of MK-0518 in Five different lots of plasma as assessed by the replicate ($n = 5$) analysis of standards

Nominal concentration (ng/mL)	Mean assayed concentration (ng/mL, $n = 5$)	Accuracy [a] (%)	Precision [b] (%)
2.0	2.0	100.0	3.2
5.0	5.0	100.0	1.0
10.0	10.3	103.0	2.6
50.0	50.8	101.6	1.4
100.0	106.8	106.8	1.1
500.0	491.5	98.3	0.7
750.0	724.7	96.6	1.7
1000.0	947.7	94.8	2.8

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

Table 2
Initial within-day analysis of MK-0518 quality control samples

	Low QC (6.0 ng/mL)	Middle QC (75 ng/mL)	High QC (750 ng/mL)
Initial Mean ($n = 5$)	6.3	80.6	739.1
Standard deviation	0.2	1.9	4.5
Precision[a] (%)	3.1	2.4	0.6
Accuracy[b] (%)	105.0	107.5	98.5

[a] Coefficent of variation.
[b] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 3
Extraction recovery and assessment of absolute matrix effects on ionization during the determination of MK-0518 in five different lots of human plasma

Standard concentration in plasma (ng/mL)	Mean extraction recovery (%) [a] ($n = 5$)	Mean absolute matrix effect (%)[b] ($n = 5$)
5.0	82.8	91.0
100.0	83.4	97.0
750.0	95.6	87.3
100 (ISTD)[c]	85.7	93.7

[a] Extraction recovery was calculated by dividing the mean peak area of analyte spiked before extraction by the respective mean peak area of analyte spiked into extracts of control plasma and multiplying by 100.
[b] Matrix effect was calculated by dividing the mean peak area of analyte spiked into control plasma extracts by the mean peak area of the neat analyte standard and multiplying by 100.
[c] ($n = 15$).

Table 4
Slopes of the standard curves in five different lots of control plasma (relative matrix effect)

Control plasma lot	Slope
A	0.0193
B	0.0192
C	0.0191
D	0.0194
E	0.0194
Mean	0.0193
Standard deviation	0.00013
Precision (%)	0.7

Table 5
Assessment of freeze–thaw (F/T) stability of MK-0518 in human plasma

Nominal concentration (ng/mL)	Control QC mean assayed concentration ($n = 5$)	Three F/T cycles mean assayed concentration ($n = 5$)	Difference from control (%)
6.0	6.0 (1.9)	6.1 (1.9)	1.6
75.0	80.8 (0.8)	80.3 (0.7)	−0.6
750.0	741.3 (1.3)	735.2 (1.6)	−0.8

Numbers is parentheses are coefficients of variation (%CV).

Table 6
Assessment of HIV heat deactivation at 56 °C for MK-0518 in human plasma

Nominal concentration (ng/mL)	QCs (control)		QCs (56 °C for 90 min)	
	Mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	Mean assayed concentration[a] (ng/mL)	Difference from control (%)
6.0	6.0 (1.9)	100.0	6.4 (8.3)	6.7
75.0	80.8 (0.8)	107.7	79.2 (1.6)	−2.0
750.0	741.3 (1.3)	98.8	736.5 (0.3)	−0.6

[a] $n = 3$.
[b] Expressed as [(mean observed concentration)/(nominal concentration)] × 100. Numbers in parentheses are coefficients of variation (%CV).

Table 7
Bench top stability of MK-0518 in human plasma

Nominal concentration (ng/mL)	Low QC (6.0 ng/mL)	Middle QC (75 ng/mL)	High QC (750 ng/mL)
Mean assayed concentration ($t = 5$ h) ($n = 3$) (ng/mL)	6.2 (3.2)	80.7 (1.9)	728.9 (0.8)
$t = 5$ h difference from nominal (%)	3.3	7.6	-2.9

Table 8
MK-0518 plasma sample dilution accuracy and precision

Dilution QCs 1:100	Calculated concentration
1	48812.4
2	49859.2
3	49833.3
4	50278.3
5	49296.0
Mean	49695.8
Standard deviation	623.3
Precision (%)	1.3
Accuracy (%)	99.4

within 7.6% of the control set. These results indicate MK-0518 is stable at room temperature in human plasma for up to 5 h. Results are shown in Table 7.

3.2.10. Sample dilution integrity

The ability to accurately dilute samples was assessed by preparing a set of human plasma samples containing MK-0518 at a concentration 50 times greater than the ULOQ (50,000 ng/mL). These dilution samples ($n = 5$) were frozen at −20 °C for at least 24 h, thawed, and diluted 1:100 with control plasma. The diluted samples were analyzed along with a standard curve. The average accuracy of the assayed concentration of these samples was 99.4% of nominal, with a CV of 1.3%. Complete results are shown in Table 8. The concentrations of all clinical samples obtained following the administration of therapeutic doses of MK-518 were below 50,000 ng/mL.

3.2.11. Processed sample stability/reinjection reproducibility

Standard curve samples and QCs ($n = 6$ per concentration) were prepared and analyzed. Following initial analysis, the samples were allowed to remain on the autosampler exposed to ambient laboratory conditions for 6 days after processing and initial injection, after which, the samples were reinjected. Assayed QC concentrations were determined based on (1) the initial injection of the standard curve samples and (2) the standard curve samples that were reinjected. The results are presented in Table 9.

Table 10
Assessment of the long-term stability of MK-0518 and $^{13}C_6$-MK-0518 (ISTD) solution stored at room temperature

	Analyte			
	MK-0518		$^{13}C_6$-MK-0518 (ISTD)	
		8 months[a]		14 months[a]
Peak areas				
1	272765.1	286223.1	19826.9	22224.5
2	273968.1	287660.8	18563.4	23184.0
3	278601.0	291995.0	17872.5	22135.4
4	276180.2	280852.6	24000.4	21443.7
5	278766.4	279210.7	19181.8	21759.5
Mean ($n = 5$)	276056.2	285188.4	19889.0	22149.4
CV (%)	1.0	1.8	5.0	2.6
Difference (%)	–	3.2[b]	–	2.1[c]

[a] Storage time.
[b] Difference after 8 months of storage at room temperature.
[c] Difference after 14 months of storage at room temperature.

3.2.12. Solution stability

The stability of MK-0518 stock solutions was evaluated by comparing the instrument response of freshly prepared standard solutions from a new standard weighing to similarly prepared solutions stored for 8 months at room temperature. The results, presented in Table 9, show a difference of 3.2% between solutions prepared 8 months apart. Internal standard ($^{13}C_6$-MK-0518) solution stability was also evaluated by comparing freshly prepared solutions from a new standard weighing to similarly prepared solutions stored for 14 months at room temperature. The results, presented in Table 10, show a difference of 2.1% between solutions prepared 14 months apart.

3.2.13. Long-term storage stability

The long-term storage stability of MK-0518 in human plasma was assessed based on the analysis of QC samples that had been prepared and stored at −20 °C for an extended period of time. The results of the assessment are shown in Table 11. Based on these results, MK-0518 was found to be stable for periods of at least 23 months in human plasma when samples are stored at −20 °C. The time period between sample collections and sample analysis did not exceed the established stability period for any of the clinical studies.

Table 9
Processed sample stability and re-injection accuracy and precision after 4 days

Nominal concentration (ng/mL)	Initial injection		Reinjection[a]		Reinjection[b]	
	Assayed concentration[c] (ng/mL)	Accuracy (%)	Assayed concentration[c] (ng/mL)	Accuracy (%)	Assayed concentration[c] (ng/mL)	Accuracy (%)
6.0	6.3 (3.1)	105.0	6.4 (3.6)	106.7	6.2 (3.7)	103.3
75.0	80.6 (2.4)	107.5	83.5 (1.1)	111.3	78.6 (1.1)	104.8
750.0	739.1 (0.6)	98.5	762.6 (1.1)	101.7	715.9 (1.1)	95.5

Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100. Numbers in parentheses are coefficients of variation (% CV).
[a] Calculated from reinjected standard curve samples.
[b] Calculated from initial injection of standard curve samples.
[c] $n = 6$.

Table 11
Long-term storage stability of MK-0518 in human plasma at −20 °C assessed by the quality control samples

Description	Low QC (6.0 ng/mL)		Middle QC (75.0 ng/mL)		High QC (750.0 ng/mL)	
	Mean ($n=5$)	Accuracy (%)	Mean ($n=5$)	Accuracy (%)	Mean ($n=5$)	Accuracy (%)
Initial analysis	5.9	98.3	75.4	100.5	716.9	95.6
QC stability[a]	6.6	109.3	79.1	105.4	722.3	96.3

[a] Analyzed after 23-month storage at −20 °C.

3.3. Clinical sample analysis

Human plasma samples were analyzed following oral doses of MK-0518. A representative post-dose chromatogram from 1 h after a 400 mg dose is shown in Fig. 3d. The sample was analyzed after a 10-fold dilution with human control plasma and the resulting concentration of MK-0518 was 5513.6 ng/mL. Mean ($n=6$ subjects) plasma concentration vs. time profiles of MK-0518 after oral administration of a single 100, 200, 400, and 800 mg dose are shown in Fig. 4.

4. Conclusions

A bioanalytical method for the determination of MK-0518 in human plasma has been validated and successfully applied to the analysis of Phase I, II, and III human clinical studies. This method has been demonstrated to be rugged and reliable.

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