BP-0001

Bioanalytical Procedure for the Determination of MK-0001 (L-000000001) in Rat Plasma

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This analytical method is based on an automated 96-well format protein precipitation extraction of drug (MK-0001) from rat plasma. MK-0001 and stable isotope labeled internal standard (SIL-MK-0001) are chromatographed using reversed phase chromatography and detected with tandem mass spectrometric detection employing a turbo ionspray (TIS) interface in the positive ion mode. The Multiple Reaction Monitoring (MRM) transitions monitored were *m/z* 457.1 → 191.0 for the drug and *m/z* 462.1 → 191.0 for the internal standard. The lower limit of quantitation (LLOQ) for this method is 0.200 ng/mL with a linear $1/x^2$ (weighting) calibration range from 0.200 to 200 ng/mL using a 20 µL plasma sample. Standard solutions are prepared in ACN/H_2O [50/50] and stored at +4°C when not in use. EDTA is used as the anticoagulant and plasma study samples are stored at -70°C.

1 INSTRUMENTATION (MAY BE SUBSTITUTED BY THEIR EQUIVALENT)

Category	Manufacturer	Components
Mass Spectrometer	Applied Biosystems	API-5500 MS/MS
LC	Waters	Acquity Binary Solvent Manager Acquity Sample Manager Acquity Sample Organizer Acquity Column Manager
Liquid Handling	Hamilton	MicroLab Star

2 SUPPLIES (MAY BE SUBSTITUTED BY THEIR EQUIVALENT)

Category (General)	Manufacturer/ Supplier
Volumetric flasks, Class A (non-disposable)	Pyrex
Graduated cylinders and bottles	Pyrex
Teflon-lined screw caps	Wheaton
Amber glass vials, various sizes	Fisher
Polypropylene tubes (15-mL and 50-mL)	Fisher
Polypropylene Cryo vials and caps (3.6-mL)	Thermo Nunc
Protein LoBind Conical Tubes (15-mL and 50-mL)	Eppendorf
Conical bottom polypropylene microcentrifuge tubes	Eppendorf Fisher
0.75-mL non-coded V-bottom pushcap microtiter tubes	Micronic
TPE capcluster microtiter tube sealing caps	Micronic Wheaton
Manual tube decapper	Micronic
LoBoRack microtiter tube racks with locking lid	Micronic
96-round deep well extraction plates, polypropylene (1.2-mL)	Arctic White LLC
96-deep well collection plates, polypropylene with tapered bottom (2-mL)	Analytical Sales & Services
96-well plate storage mats (covers)	Phenomenex
Pierceable TPE cap mats	Micronic
ArctiSeal 96 well square silicone/PTFE Coating (Plate Mats)	Arctic White LLC
Advantage 9mm, 12x32, TPX Wide Mouth Twist MicroVial w/Built-In 100 µL Volume Insert	Analytical Sales & Services
Certified 9mm Blue Twist Cap w/ PTFE/Silicone Liner	Analytical Sales & Services
Weighing Spatula	Fisher
Aluminum weighing boats	Fisher
Tweezers	Fisher
Acquity UPLC® HSS T3 1.8 µm 2.1 x 50mm Column (part number: 186003538)	Waters

Category (Equipment)	Manufacturer
Microbalance	Sartorius
Analytical Balance	Sartorius Ohaus
Mini Vortexer	Fisher Scientific Industries
Multi-tube vortex mixer	VWR Fisher
Thermomixer	Eppendorf
Microplate De-Froster	Integrated Technologies Ltd.
Refrigerated centrifuge	Beckman Eppendorf
Rotator	Glas-Col
Column Heater	Waters Acquity
Plate sealer	Corning Matrix

Category (Pipettes)	Manufacturer
Repeater Plus Pipettes	Eppendorf
Combitips (various volumes)	Eppendorf
Adjustable Pipettes	Labsystem Rainin Fisher Eppendorf Thermo
Pipette Tips	BioHit Thermo Fisher Eppendorf

Category (Automation Supplies)	Manufacturer
Reagent Troughs	Hamilton
Automated Workstation Tips	Hamilton

3 REFERENCE STANDARDS

Category	Parent Drug (Analyte)	Internal Standard (IS)
Analyte / L-Number	MK-0001 / L-000000001	[$^{13}C_2$, D3]MK-0001/ L-000000001
Form	001H	003M
Molecular Weight (free form)	456.54	461.536
Watson ID	MK-0001	SIL-MK-0001

4 BIOLOGICAL MATRICES

Matrix	Species	Anticoagulant	Supplier
Plasma	Rat	EDTA	BioIVT SALAR in house

5 REAGENTS (MAY BE SUBSTITUTED BY THEIR EQUIVALENT)

Reagent	Abbreviation	Provider
Deionized water (Milli-Q)	H_2O	Millipore
Acetonitrile (HPLC or LC/MS)	ACN	Fisher
Isopropanol (Chromasolv)	IPA	Sigma-Aldrich
Acetone (Chromasolv)	ACET	Sigma-Aldrich
Propionic Acid	PA	Fisher
0.1% Formic Acid in Acetonitrile (LC/MS)	0.1% FA in ACN or ACN with 0.1% FA	Fisher
0.1% Formic Acid in Water (LC/MS)	0.1% FA in H_2O or H_2O with 0.1% FA	Fisher
Dimethyl Sulfoxide (Chromasolv Plus)	DMSO	Sigma-Aldrich
Formic Acid (98+% pure)	FA	Acros
Water Solution contains 0.1% (v/v) formic acid, 20% (v/v) acetonitrile	ACN/H_2O/FA [20/80/0.1]	Sigma-Aldrich
Acetonitrile Solution contains 40.0% 2-propanol, 0.05% formic acid, 10.0% acetone	ACN/IPA/Acetone/FA [50/40/10/0.05]	Sigma-Aldrich

6 SOLUTIONS (VOLUME OF SOLUTION PREPARED MAY BE SCALED AS NEEDED)

Solution Name	Preparation Summary	Use	Storage
ACN/H_2O [50/50]	500 mL ACN + 500 mL H_2O. Mix.	Diluent	Ambient
0.1% FA in ACN	Purchased or 1 mL FA + 1000 mL ACN. Mix.	Extraction Solvent	Ambient
0.1% PA in H_2O	1 mL PA + 1000 mL H_2O. Mix.	Mobile Phase A	Ambient
0.1% PA in ACN	1 mL PA + 1000 mL ACN. Mix.	Mobile Phase B	Ambient
ACN/H_2O/FA [20/80/0.1]	Purchased or 200 mL 0.1% FA in ACN + 800 mL 0.1% FA in H_2O. Mix.	Weak Wash	Ambient
ACN/IPA/Acetone/FA [50/40/10/0.05]	Purchased or 500 mL ACN + 400 mL IPA + 100 mL Acetone + 0.5 mL FA. Mix.	Strong Wash	Ambient
ACN/H_2O [10/90]	100 mL ACN + 900 mL H_2O. Mix.	Seal Wash	Ambient
0.1% FA in H_2O	Purchased or 1 mL FA + 1000 mL H_2O. Mix.	Post Extraction Dilution Solvent	Ambient

7 MK-0001 STANDARD PREPARATION

A. *Stock Standard Solution*

Weigh the compound and transfer into an amber glass vial. Dissolve in DMSO to make a 1.00 mg/mL free form stock solution while correcting for potency (e.g., purity, residual solvents, and excess water content) and salt factor, as appropriate. Mix well. Store refrigerated (+4ºC).

B. *Working Standard Solutions*

Using adjustable pipettes, transfer the spike volume of each standard indicated in the table below into individual Protein LoBind Conical Tubes. Dilute to appropriate volume with diluent (ACN/H_2O [50/50]). Mix well. Store refrigerated (+4ºC).

Working Standard Solution ID	Spiking Standard Solution Conc. (ng/mL)	Spike Volume (mL)	Diluent Volume (mL)	Final Volume (mL)	Working Standard Conc. (ng/mL)	Plasma Standard Conc.[a] (ng/mL)
STD A	1,000,000 (Stock)	0.200	9.80	10.0	20,000	N/A
STD 8	20,000 (STD A)	1.00	9.00	10.0	2,000	200
STD 7	20,000 (STD A)	0.800	9.20	10.0	1,600	160
STD 6	2,000 (STD 8)	2.00	8.00	10.0	400	40.0
STD 5	2,000 (STD 8)	1.00	9.00	10.0	200	20.0
STD 4	2,000 (STD 8)	0.200	9.80	10.0	40.0	4.00
STD 3	200 (STD 5)	0.400	9.60	10.0	8.00	0.800
STD 2	200 (STD 5)	0.200	9.80	10.0	4.00	0.400
STD 1	200 (STD 5)	0.100	9.90	10.0	2.00	0.200

[a] A 20 µL spike of the working standards into 180 µL plasma yields the plasma standard concentration.

8 MK-0001 QUALITY CONTROL (QC) SAMPLE PREPARATION

A. *Stock QC Solution*

Weigh the compound and transfer into an amber glass vial. Dissolve in DMSO to make a 1.00 mg/mL free form stock solution while correcting for potency (e.g., purity, residual solvents, and excess water content) and salt factor, as appropriate. Mix well. Store refrigerated (+4ºC).

B. *Working QC Solutions*

Using adjustable pipettes, transfer the volumes of each QC indicated in the table below into Protein LoBind Conical Tubes. Dilute to appropriate volume with diluent (ACN/H_2O [50/50]). Mix well. Store refrigerated (+4ºC).

Working QC Solution ID	Spiking QC Solution Conc. (ng/mL)	Spike Volume (mL)	Diluent Volume (mL)	Final Volume (mL)	Working QC Solution Conc. (ng/mL)
-A-	1,000,000 (Stock)	0.200	1.80	2.00	100,000
-B-	100,000 (QC A)	0.750	9.25	10.0	7,500
-C-	7,500 (QC B)	0.360	8.64	9.00	300
-D-	300 (QC C)	1.00	9.00	10.0	30
-E-	300 (QC C)	0.300	8.70	9.00	10

C. *Matrix QCs*

To a polypropylene tube, add the designated spiking volume of appropriate spiking solution to blank matrix. Cap the tube and briefly vortex. Aliquot 0.150 mL into micronic tubes, cap, and store at -70ºC. The aliquot volume and QCs volume prepared may be scaled as needed. Matrix QCs may be used from freshly prepared or frozen samples.

Matrix QC ID	Spiking QC Solution Conc. (ng/mL)	Spike Volume (mL)	Matrix Volume (mL)	Final Volume (mL)	Matrix QC Conc. (ng/mL)
HIGH	7,500 (QC B)	0.300	14.7	15.0	150
MID	300 (QC C)	0.300	14.7	15.0	6.00
LOW	30.0 (QC D)	0.300	14.7	15.0	0.600
LLOQ	10.0 (QC E)	0.300	14.7	15.0	0.200

9 INTERNAL STANDARD (IS) PREPARATION

1. Stock Solution: (1.00 mg/mL SIL-MK-0001 stock solution): Weigh the compound and transfer into an amber glass vial. Dissolve in DMSO to make a 1.00 mg/mL stock solution while correcting for potency and salt factor, if available. Mix well. Store refrigerated (+4ºC).
2. Intermediate Internal Standard Solution (10,000 ng/mL): Pipet 100 µL of the internal standard stock solution into an amber glass vial and add 9.90 mL of ACN/H_2O [50/50]. Mix well. Store refrigerated (+4ºC).
3. Working Internal Standard Solution (10.0 ng/mL): Pipet 50.0 µL of the intermediate internal standard solution into an amber glass vial and add 49.95 mL of ACN/H_2O [50/50]. Mix well. Store refrigerated (+4ºC).

10 UNKNOWN SAMPLE PREPARATION

The glassware, reagents, and dilutions described in this procedure should serve as a guide and may be substituted for their equivalent when necessary to obtain similar results.

Blank plasma:

- Thaw, vortex and centrifuge for 5 minutes at approximately 2800 rpm
- Store frozen (-20ºC) when not in use.

Biological plasma samples (e.g., unknown samples, dilution check QCs):

- Allow samples to thaw completely
- Under ambient temperature, vortex samples for 5 minutes at 1200 rpm and centrifuge for 1 minute at approximately 2800 rpm
- Aliquot samples and control blank plasma as appropriate using the dilution scheme below, mix well:

Initial Dilution Factor	Sample Volume, µL	Blank Plasma, µL
5	12	48
10	12	108
20	12	228
40	12	234

- After dilution, diluted samples can be analyzed immeditately or stored at -70ºC before analysis

11 PROCEDURE

The glassware, reagents, and dilutions described in this procedure should serve as a guide and may be substituted for their equivalent when necessary to obtain similar results.

Blank plasma:

Thaw, vortex and centrifuge for 5 minutes at approximately 2800 rpm

Biological plasma samples (e.g., unknown samples, diluted unknown samples, matrix QCs):

1. Allow samples to thaw completely
2. Vortex samples for 5 minutes at 1200 rpm
3. Centrifuge at approximately 1200 RPM for 1 minute at ambient temperature.

A. Preparation of Calibration Standards, Quality Controls, Blanks, and Unknown Samples

Step	Activity
1	Prepare the standard spiked blank plasma as follows: To a microtiter tube, add: • 180 µL of blank plasma • 20 µL of the appropriate MK-0001 working standard solution
2	Cap the microtiter tubes and mix well. Remove any air bubbles.
3	Add to a 96-round deep well extraction plate (1.2 mL) the following: For Calibration Standards: • 20 µL of appropriate plasma standards (from Step 2 above) • 20 µL of IS working standard solution For Blank Samples: • 20 µL of appropriate blank plasma • 20 µL of Diluent For Standard 0 (IS Blank Sample): • 20 µL of appropriate blank plasma • 20 µL of IS working standard solution For Unknown, Diluted Unknown and Quality Control Samples*: • Appropriate volume of unknown sample, diluted unknown or matrix QC (and blank plasma if needed) for a final volume of 20 µL • 20 µL of IS working standard solution
4	Add 100 µL of crash solvent (0.1% FA in ACN) to extraction plate.
5	Seal the deep well extraction plate with appropriate plate mat.
6	Vortex for 60 seconds using a multi-vortexer at Speed 6.
7	Centrifuge the plate at 4000 RPM for 10 minutes at ambient temperature.
8	Transfer 50 µL of supernatant to a new deep well collection plate (2-mL).
9	Dilute the supernatant with 50 µL of 0.1% FA in H2O.
10	Seal the deep well collection plate with appropriate storage mat.
11	Vortex for 2 minutes using a multi-vortexer at Speed 6.
12	Centrifuge the plate at 1200 RPM for 1 minute at ambient temperature.
13	Place the deep well plate in the autosampler and inject appropriate volume.

* Note: if double dilution is required, add first to a 96-round deep well extraction plate (1.2 mL) plate:
a. Appropriate volume of unknown or diluted unknown samples.
b. Appropriate volume of blank plasma.
c. Vortex for 120 seconds at 1100 rpm at ambient temperature then process following Procedure A beginning with Step 3 for Unknown, *Diluted Unknown and Quality Control Samples.*

Note: The following dilution schemes may be used as a guide.

Single Dilutions		
Dilution Factor	**Sample Volume, µL**	**Blank Plasma, µL**
2	10	10
5	4	16

Single Dilutions after the Initial Sample Dilution in Section 10		
Dilution Factor**	**1:X Diluted Sample Volume, µL**	**Blank Plasma, µL**
2	10	10
5	4	16

Double Dilutions after the Initial Sample Dilution in Section 10			
Dilution Factor**	**1:X Diluted Sample Volume, µL**	**Blank Plasma, µL**	**Dilution 1 Dilution 2**
10	20 20	180 0	10 1
20	20 10	180 10	10 2
50	20 4	180 16	10 5
60	10 10	290 10	30 2
80	10 5	190 15	20 4
100	10 4	190 16	20 5
200	10 4	390 16	40 5

** This dilution factor does not account for the initial dilution in the Section 10.

12 OPERATING PARAMETERS

UPLC	Settings	
Column	Acquity UPLC® HSS T3 1.8 µm 2.1 x 50mm Column	
Loop Option	Partial Loop	
Needle Type	PEEKSIL (Peptide Needle)	
Elution	Gradient, see below for details	
Flow Rate	0.6 mL/min	
Mobile Phase A	0.1% PA in H_2O	
Mobile Phase B	0.1% PA in ACN	
Injection Volume (injection volume may be adjusted to obtain adequate response)	5 µL	
Column Temperature	Ambient	
Autosampler Temperature	15 ºC	
Divert	Waste (A): Initial Detector (B): 1.10 min Waste (A): 2.00 min	
Run Time	3.0 minutes	
Retention Time	MK-0001: 1.60 min.	SIL-MK-0001: 1.60 min.

UPLC Profile	Time (min)	Flow Rate (mL/min)	% A (0.1% PA in H_2O)	% B (0.1% PA in ACN)	Curve
	Initial	0.600	60.0	40.0	Initial
	0.20	0.600	60.0	40.0	6
	2.20	0.600	50.0	50.0	6
	2.21	0.600	5.0	95.0	6
	2.70	0.600	5.0	95.0	6
	2.71	0.600	60.0	40.0	6
	3.00	0.600	60.0	40.0	6

Wash Type	Wash Solvent	Wash Volume (µL)	Wash Time (min)
Weak Wash	ACN/H_2O/FA [20/80/0.1]	600	N/A
Strong Wash	ACN/IPA/Acetone/FA [50/40/10/0.05]	200	N/A
Seal Wash	ACN/H_2O [10/90]	N/A	5.00

MS	Settings
Ion Source	Turbo Ionspray
Ion Mode	Positive
Q1/Q3 Resolutions	unit/unit
Scan Type	MRM
Ionization potential (IS)	5500 V
Temperature	550°C
Curtain Gas – N_2*	40
GS 1 – N_2*	50
GS 2 – N_2*	60
CAD – N_2*	9
MR pause between mass range	5.0070 ms
MS settling time	0.0000 ms

Ions Monitored*	Q1 *m/z*	Q3 *m/z*	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
MK-0001	457.1	191.0	100.00	50	10	25	14
SIL-MK-0001	462.1	191.0	100.00	50	10	25	14

*Parameters may be adjusted to obtain adequate response.

13 SYSTEM SUITABILITY

When assaying biological extracts:

An extracted system suitability sample at the LLOQ will be injected prior to sample analysis to ensure that the LC-MS/MS system is functioning as intended. The results should meet the following minimum acceptance criteria for system performance, or the samples cannot be injected, unless a valid scientific reason is observed and documented (e.g., signal : noise ratio ≥20:1, peak height).

Analyte	Peak Height	Retention time (min)
MK-0001 (0.2 ng/mL plasma STD)	≥800 cps	1.60 ± 0.25

When conducting stock or working solution stability assessments:

A neat 2.00 ng/mL solution of MK-0001 will be injected prior to initiating stock and working solution stability sample analysis to ensure that the LC-MS/MS system is functioning as intended.

1. Spike 20 µL of STD 1 and 20 µL of SIL-MK-0001 into a 3.6-mL nunc tube. Mix well.
2. Add 100 µL of 0.1% FA in ACN to the sample.
3. Dilute the sample with 140 µL of 0.1% FA in H_2O.
4. Cap the tube and briefly vortex to mix.
5. Transfer the solution into the sample injection plate.
6. Place the injection plate in the autosampler and inject appropriate volume.

Analyte	Peak Height	Retention time (min)
MK-0001	≥8000 cps	1.60 ± 0.25

14 SOFTWARE AND CALCULATION

1. Raw peak areas generated in the SCIEX Analyst® (ver. 1.6.2 or higher) software package are commonly exported into the WATSON system for quantitation.
2. Daily standard curves are constructed from peak area ratios of MK-0001 to internal standard versus the nominal concentrations of standards.
3. Unknown sample concentrations are calculated from the equation $y = mx + b$, by weighted ($1/x^2$) linear least square regression of the calibration line constructed by plotting the peak area ratio (drug to internal standard) of the standard curve samples *versus* nominal drug concentration.

Jieutonne Archer
James Marr
Tonya Jackson
James Schiller

APPENDIX A

GENERAL LIST OF USED ACRONYMS

ACET	Acetone
ACN	Acetonitrile
Ambient	Room Temperature
APCI	Atmospheric Pressure Chemical Ionization
BP	Bioanalytical Procedure
°C	Degree Celsius
CAD	Collisionally Activated Dissociation
Conc.	Concentration
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine tetra-acetic acid
FA	Formic Acid
GLP	Good Laboratory Practice
GS	Gas Source (for MS)
H_2O	Water
HPLC	High Performance Liquid Chromatography
IPA	Isopropanol
IS, ISTD	Internal Standard
ISV	Ion Spray Voltage
L	Volume Expressed in Liters
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometer
LLQ/LLOQ	Lower Limit of Quantitation
MeOH	Methanol
mg	Weight expressed in milligrams
min	Time expressed in minutes
mL	Volume expressed in milliliters
MRL	Merck Research Laboratories
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometer
MTBE	Methyl Tert Butyl Ether
N_2	Nitrogen
N/A, NA	Not Applicable
NDS	Nonclinical Drug Safety
PA	Propionic Acid
PBMC	Peripheral Blood Mononuclear Cells
PCD	Preclinical Development
PN	Part Number
PPDM	Pharmacokinetics, Pharmacodynamics, and Drug Metabolism
QC	Quality Control Sample

APPENDIX A (Cont.)

QS	Quantity Sufficient
rpm	Revolutions per minute
s	Time expressed in seconds
SALAR	Safety Assessment and Laboratory Animal Resources
sig. fig.	Significant Figures
SIL	Stable-labeled internal Standard
SOP	Standard Operating Procedure
STD	Standard
TFA	Tri-fluoroacetic acid
TIS	Turbo Ionspray
ULQ/ULOQ	Upper Limit of Quantitation
UPLC	Ultra Performance Liquid Chromatography
Vol.	Volume
WP	West Point

DOCUMENT HISTORY

Document Version	Date Updated	Purpose
1.0	Original	Original Document