INTRODUCTION

OBJECTIVE

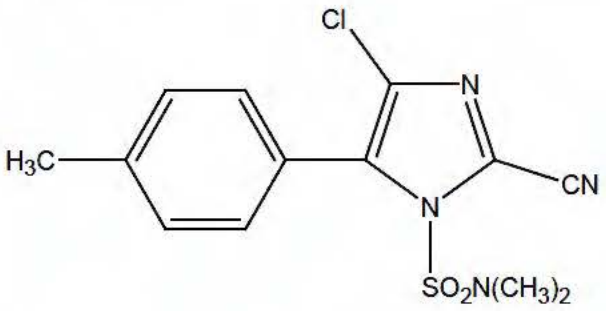
The objective of this study was to perform an independent laboratory validation (ILV) of an extraction method for Cyazofamid (IKF-916), and four metabolites (CCIM, CCIM-AM, CTCA, and CCBA) in surface and ground water.

CONDUCT OF THE STUDY

The study was conducted at Concord Biosciences, LLC in accordance with protocol "Independent Laboratory Validation – Determination of Residues of Cyazofamid (IKF-916), and Four Metabolites (CCIM, CCIM-AM, CTCA, and CCBA) in Surface Water and Ground Water Using LC-MS/MS", Document Number 035869-0. The protocol is given in Appendix A.

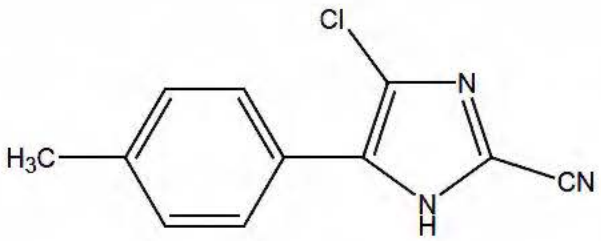
TEST SUBSTANCE

- **Cyazofamid (IKF-916)**



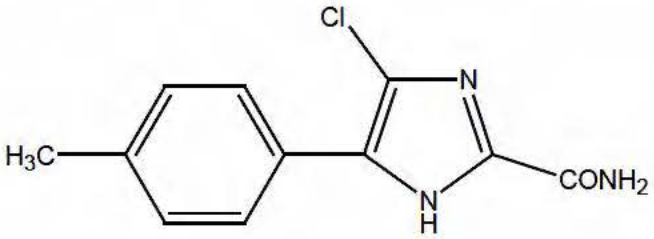
Common Name:	Cyazofamid or IKF-916
Chemical Name:	4-Chloro-2-cyano-*N,N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide
CAS No:	120116-88-3
Molecular Formula:	$C_{13}H_{13}ClN_4O_2S$
Molecular Weight:	324.79 g/mol
Lot No.:	9506
Purity:	95.6%
Expiration Date:	April 08, 2020
Storage:	~ -20 °C
Concord Biosciences Inventory No.:	CS_23111

- **CCIM**



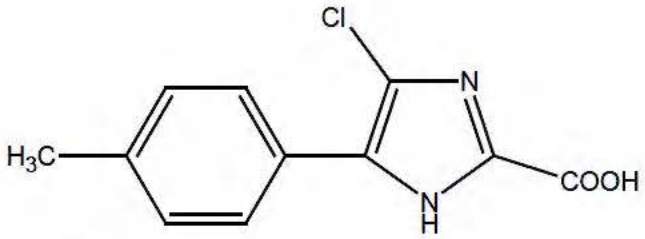
Common Name:	CCIM
Chemical Name:	4-Chloro-5-*p*-tolylimidazole-2-carbonitrile
Molecular Formula:	$C_{11}H_8ClN_3$
Molecular Weight:	217.65 g/mol
Lot No.:	20150916
Purity:	99.6%
Expiration Date:	March 23, 2021
Storage:	~ 4 ± 4 °C, in the dark
Concord Biosciences Inventory No.:	CS_23112

- **CCIM-AM**



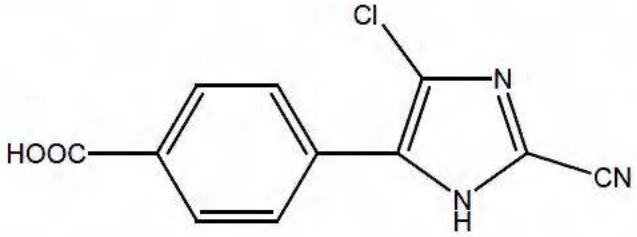
Common Name:	CCIM-AM
Chemical Name:	4-Chloro-5-*p*-tolylimidazole-2-carboxamide
Molecular Formula:	$C_{11}H_{10}ClN_3O$
Molecular Weight:	235.67 g/mol
Lot No.:	9809
Purity:	99.7%
Expiration Date:	August 19, 2020
Storage:	~ -20 °C
Concord Biosciences Inventory No.:	CS_23113

- **CTCA**



Common Name:	CTCA
Chemical Name:	4-Chloro-5-*p*-tolylimidazole-2-carboxylic acid
Molecular Formula:	$C_{11}H_9ClN_2O_2$
Molecular Weight:	236.65 g/mol
Lot No.:	0505
Purity:	99.3%
Expiration Date:	September 03, 2018
Storage:	~ -20 °C
Concord Biosciences Inventory No.:	CS_23114

- **CCBA**



Common Name:	CCBA
Chemical Name:	4-(4-Chloro-2-cyanoimidazol-5-yl)benzoic acid
Molecular Formula:	$C_{11}H_6ClN_3O_2$
Molecular Weight:	247.64 g/mol
Lot No.:	0001
Purity:	99.6%
Expiration Date:	March 17, 2022
Storage:	~ -20 °C
Concord Biosciences Inventory No.:	CS_23115

TEST SYSTEM

- **Ground and Surface Water**

The ground water (Concord inventory number EFS-625) was collected from 3254 Callender Road, Rock Creek, OH. The pond water (surface water, Concord Inventory number EFS-615) was collected from Smokey Oaks Pond. The ground water and surface water characterization data is summarized in Appendix C.

MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

- Acetonitrile: Fisher Scientific Optima Grade and Sigma Aldrich, HPLC grade
- Water: Fisher Scientific and Sigma Aldrich, HPLC Grade
- Dichloromethane: Fisher Scientific Reagent Grade or equivalent
- Ammonium Acetate: Sigma-Aldrich
- QuEChERs mix, Chromabond and Phenomenex
- Formic Acid: Sigma Aldrich, Reagent Grade
- Ammonium Acetate: Sigma Aldrich, Trace metals basis
- **Mobile Phase A (5mM Ammonium acetate in 0.1% Formic Acid in water):** For every liter prepared, add 0.385 grams of ammonium acetate to 1000 mL of water in a glass bottle and add 1.0 mL of formic acid. Mix the solution properly and store at ambient conditions. The solution expires after 3 months.
- **Mobile Phase B (0.1% Formic Acid in methanol)**: For every liter prepared, add 1.0 mL of formic acid to 1000 mL of methanol in a glass bottle. Mix properly and store at ambient conditions. The solution expires after 3 months.
- **Water:Acetontrile (50:50)**: To a 1000 mL graduated cylinder, add 500 mL of water and 500 mL of acetonitrile, and then transferr to a 1000 mL carboy bottle. The bottle was capped and inverted several times for uniform mixing and stored at ambient conditions. The solution expired after 3 months.
- **Needle Rinse**: 33/33/33/ + 1% Formic acid (2-Propanol/Acetone/Acetonitrile)

  Combine 20 mL formic acid with 660 mL 2-propanol, 660 mL acetone and 660 mL acetonitrile in a bottle. Mix well. Store at room temperature or lower. Expiration is 3 months from the date of preparation. Preparation can be scaled up or down proportionately.

PREPARATION OF TEST SUBSTANCE STOCK SOLUTIONS

Stock solution was prepared in duplicate using appropriate weight for lot purity correction. All solutions were stored at frozen temperature conditions (e.g., -5 to -35°C).

The following series of primary stock solutions and working solutions were prepared in 20-mL glass vials. Solvents were dispensed using volumetric or calibrated pipettes. Dilutions were made as described below.

Primary Stock Solutions (1000 µg/mL) [SA01 and CA01] for IKF-916, CCIM, CCIM-AM, CCBA and CTCA

IKF-916 Stock Solution
Using an analytical balance, 10.70 mg and 10.55 mg of the standard was weighed, respectively into10-mL glass vialsthe actual weight was recorded (corrected for purity) and diluted to volume with acetonitrile. The exact concentration of the stock solution was calculated. The SA01 was used for the preparation of the secondary stock solution and subsequent preparation of calibrations standards. The CA01 was used for the preparation of the secondary stock solution and fortification solutions.

CCIM Stock Solution
Using an analytical balance, 10.45 mg and 10.17 mg of the standard was weighed, respectively into 10-mL glass vials the actual weight was recorded (corrected for purity) and diluted to volume with acetonitrile. The exact concentration of the stock solution was calculated. The SA01 was used for the preparation of the secondary stock solution and subsequent preparation of calibration standards. The CA01 was used for the preparation of secondary stock solution and fortification solutions.

CCIM-AM Stock Solution
Using an analytical balance, 10.11 mg and 10.25 mg of the standard was weighed, respectively into 10-mL glass vials the actual weight was recorded (corrected for purity) and diluted to volume with acetonitrile. The exact concentration of the stock solution was calculated. The SA01 was used for the preparation of the secondary stock solution and subsequent preparation of calibration standards. The CA01 was used for the preparation of secondary stock solution and Fortification solutions.

CCBA Stock Solution
Using an analytical balance, 10.51 mg and 10.16 mg of the standard was weighed, respectively into 10-mL glass vials the actual weight was recorded (corrected for purity) and diluted to volume with acetonitrile. The exact concentration of the stock solution was calculated. The SA01 was used for the preparation of the secondary stock solution and subsequent preparation of calibration standards. The CA01 was used for the preparation of secondary stock solution and fortification solutions.

CTCA Stock Solution
Using an analytical balance, 10.11 mg and 10.25 mg of the standard was weighed, respectively into 10-mL glass vials the actual weight was recorded (corrected for purity) and diluted to volume with acetonitrile. The exact concentration of the stock solution was calculated. The SA01 was used for the preparation of the secondary stock solution and subsequent preparation of calibration standards. The CA01 was used for the preparation of secondary stock solution and fortification solutions.

Preparation of Secondary Stock Solutions (100 µg/mL) [SA02 and CA02]

IKF-916:
SA02 100 µg/mL solution was prepared by adding 0.978 mL of the 1,023 µg/mL stock solution, into a glass vial containing 9.022 mL of acetonitrile. Vortex well to mix.

CA02 100 µg/mL solution was prepared by adding 0.992 mL of the 1.009 µg/mL stock solution into a glass vial containing 9.008 mL of acetonitrile. Vortex well to mix.

CCIM:
SA02 100 µg/mL solution was prepared by adding 0.961 mL of the 1,041 µg/mL solution, into a scintillation vial containing 9.039 mL of acetonitrile. Vortex well to mix.

CA02 100 µg/mL solution was prepared by adding 0.987 mL of the 1,013 µg/mL stock solution into a glass vial containing 9.013 mL of acetonitrile. Vortex well to mix.

CCIM-AM:
SA02 100 µg/mL solution was prepared by adding 0.992 mL of the 1,008 µg/mL solution, into a scintillation vial containing 9.008 mL of acetonitrile. Vortex well to mix.

CA02 100 µg/mL solution was prepared by adding 0.979 mL of the 1,022 µg/mL stock solution into a glass vial containing 9.021 mL of acetonitrile. Vortex well to mix.

CCBA:
A 100 µg/mL solution was prepared by adding 0.955 mL of the 1,047 µg/mL solution, into a scintillation vial containing 9.045 mL of acetonitrile. Vortex well to mix.

CA02 100 µg/mL solution was prepared by adding 0.988 mL of the 1,012 µg/mL stock solution into a glass vial containing 9.012 mL of acetonitrile. Vortex well to mix.

CTCA:
SA02 100 µg/mL solution was prepared by adding 0.996 mL of the 1,004 µg/mL solution, into a scintillation vial containing 9.004 mL of acetonitrile. Vortex well to mix.

CA02 100 µg/mL solution was prepared by adding 0.983 mL of the 1,018 µg/mL stock solution into a glass vial containing 9.017 mL of acetonitrile. Vortex well to mix.

PREPARATION OF FORTIFICATION STANDARD SOLUTIONS (MIX-3)

Preparation of 1000/5000 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 1000/5000 ng/mL fortification solution was prepared by mixing 0.1 mL of 100 µg/mL IKF-916, 0.5 mL of 100 µg/mL CCIM and 0.5 mL of 100 µg/mL CCIM-AM and then adding 8.9 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 100/500 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 100/500 ng/mL fortification solution was prepared by mixing 1 mL of 1000/5000 ng/mL (IKF-916/Metabolites CCIM and CCIM-AM) of Mix-3 standard and then adding 9.0 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 10/50 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 10/50 ng/mL fortification solution was prepared by mixing 1 mL of 100/500 ng/mL (IKF-916/Metabolites CCIM and CCIM-AM) of Mix-3 standard and then adding 9.0 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

PREPARATION OF CALIBRATION STANDARD SOLUTIONS (MIX-3)

Preparation of 1000/5000 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 1000/5000 ng/mL calibration solution was prepared by mixing 0.1 mL of 100 µg/mL IKF-916, 0.5 mL of 100 µg/mL CCIM and 0.5 mL of 100 µg/mL CCIM-AM and then adding 8.9 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 100/500 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 100/500 ng/mL calibration solution was prepared by mixing 1 mL of 1000/5000 ng/mL (IKF-916/Metabolites CCIM and CCIM-AM) of Mix-3 standard and then adding 9.0 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 10/50 ng/mL IKF-916/ Metabolites CCIM and CCIM-AM intermediate mixed solution (Mix-3)

A 10/50 ng/mL calibration solution was prepared by mixing 1 mL of 100/500 ng/mL (IKF-916/Metabolites CCIM and CCIM-AM) of Mix-3 standard and then adding 9.0 mL of acetonitrile to bring the final volume to 10 mL in a glass vial. Mixed well by vortexing.

The volume and the concentration of the solutions used in the calibration standard solutions are given below.

Std. ID	Conc. of Source Solution (ng/mL)	Source Solution Vol. (mL)	ACN: Water (50:50) Vol. (mL)	Prepared Conc. (ng/mL)
Std-8	100/500	1.00	9.00	10/50.0
Std-7	100/500	0.750	9.25	7.5/37.5
Std-6	100/500	0.500	9.50	5.0/25.0
Std-5	100/500	0.200	9.80	2.0/10
Std-4	100/500	0.100	9.90	1.0/5.0
Std-3	5.0/25.0	1.00	9.00	0.5/2.5
Std-2	2.0/10	1.00	9.00	0.2/1.0
Std-1	1.0/5.0	1.00	9.00	0.1/0.5

PREPARATION OF FORTIFICATION STANDARD SOLUTIONS (MIX-2)

Preparation of 5000 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 5000 ng/mL fortification solution was prepared by mixing 0.5 mL of 100 µg/mL CCBA and 0.5 mL of 100 µg/mL CTCA and then add 9.0 mL of acetonitrile to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 500 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 500 ng/mL fortification solution was prepared by mixing 1 mL of 5000 ng/mL (CCBA and CCTA) of Mix-2 standard and then add 9.0 mL of ACN:Water (50:50) to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 50 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 50 ng/mL fortification solution was prepared by mixing 1 mL of 500 ng/mL (CCBA and CCTA) of Mix-2 standard and then add 9.0 mL of ACN:Water (50:50) to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.

PREPARATION OF CALIBRATION STANDARD SOLUTIONS (MIX-2)

Preparation of 5000 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 5000 ng/mL calibration solution was prepared by mixing 0.5 mL of 100 µg/mL CCBA and 0.5 mL of 100 µg/mL CTCA and then add 9.0 mL of acetonitrile to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 500 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 500 ng/mL calibration solution was prepared by mixing 1 mL of 5000 ng/mL (CCBA and CCTA) of Mix-2 standard and then add 9.0 mL of ACN:Water (50:50) to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.

Preparation of 50 ng/mL CCBA and CCTA intermediate mixed solution (Mix-2)

A 50 ng/mL calibration solution was prepared by mixing 1 mL of 500 ng/mL (CCBA and CCTA) of Mix-2 standard and then add 9.0 mL of ACN:Water (50:50) to bring the final volume of 10 mL in a glass vial. Mixed well by vortexing.The volume and the concentration of the solutions used in the calibration standard solutions are given below:

Std. ID	Conc. of Source Solution (ng/mL)	Source Solution Vol. (mL)	ACN: Water (50:50) Vol. (mL)	Prepared Conc. (ng/mL)
Std-8	5000	1.00	9.00	500
Std-7	5000	0.750	9.25	375
Std-6	5000	0.500	9.50	250
Std-5	5000	0.200	9.80	100
Std-4	5000	0.100	9.90	50
Std-3	250	1.00	9.00	25
Std-2A	500	0.300	9.70	15
Std-2	100	1.00	9.00	10
Std-1	50	1.00	9.00	5

The above prepared calibration standard solutions were used to make matrix-matched calibration standard solutions. The matrix-matched calibration standard curve was prepared by adding 50 µL of each calibration standard into separate HPLC vials and adding 450 µL of control matrix (**Note:** Extra control matrix samples were prepared and used to prepare matrix-matched calibration standard curve).

All calibration and fortification solutions were stored frozen (~ -20 °C) when not in use.

ANALYTICAL METHODOLOGY

Two different extraction procedures were used to extract IKF-916 and its metabolites. IKF-916, CCIM and CCIM-AM (Mix-3) were extracted with a liquid-liquid extraction method, whereas CCBA and CCTA were extracted using a QuEChERs method.

LIQUID-LIQUID EXTRACTION OF GROUND WATER/SURFACE WATER FOR IKF-916, CCIM AND CCIM-AM (MIX-3)

1) Aliquot 20 mL of water (ground and/or surface) sample into each separatory funnel.

2) Fortify control water samples according to the fortification table below.

Standard Name	Fortification Conc. (ppb)	Fortification Volume of mixed standard solution
Control-1	NA	NA
Control-1	NA	NA
LOQ-1	0.1/0.5	200 µL of 10/50 ng/mL
LOQ-2	0.1/0.5	200 µL of 10/50 ng/mL
LOQ-3	0.1/0.5	200 µL of 10/50 ng/mL
LOQ-4	0.1/0.5	200 µL of 10/50 ng/mL
LOQ-5	0.1/0.5	200 µL of 10/50 ng/mL
10X LOQ-1	1.0/5.0	200 µL of 100/500 ng/mL
10X LOQ-2	1.0/5.0	200 µL of 100/500 ng/mL
10X LOQ-3	1.0/5.0	200 µL of 100/500 ng/mL
10X LOQ-4	1.0/5.0	200 µL of 100/500 ng/mL
10X LOQ-5	1.0/5.0	200 µL of 100/500 ng/mL

Step	Extraction Procedure
1	Add 10 mL of dichloromethane to each sample
2	Shake the separatory funnels for about 2 minutes
3	Transfer the dichloromethane layer into 15 mL glass tube, put into TurboVap under nitrogen set at 40°C
4	Add another 10 mL of dichloromethane to the sample. Repeat steps 2 and 3. Combine dichloromethane extracts.
5	Evaporate to dryness in TurboVap under nitrogen set at 40 ºC. Reconstitute with 2 mL of 50:50 ACN:Water.
6	Transfer to LC vials for control and LOQ samples and submit for LC-MS/MS analysis. For 10X-LOQ samples, aliquot 0.5 mL of extract and add 0.5 mL of 50:50 ACN:Water in a HPLC vial. Mix well by vortexing and submit for LC-MS/MS analysis.

QuEChERS extraction procedure of Ground water/surface water for CCBA and CTCA (Mix-2)

1. Transfer 10 mL of each water (ground water/surface water) sample into separate 50 mL centrifuge tubes.

2. Fortify control water samples according to the Fortification Table below.

Fortification Table

Sample ID	Fortification Conc. (ppb)	Fortification Volume of mixed standard solution
Control-1	N/A	N/A
Control-2	N/A	N/A
LOQ-1	0.5	100 µL of 50ng/mL
LOQ-2	0.5	100 µL of 50 ng/mL
LOQ-3	0.5	100 µL of 50ng/mL
LOQ-4	0.5	100 µL of 50 ng/mL
LOQ-5	0.5	100 µL of 50ng/mL
10x LOQ-1	5.0	100 µL of 500 ng/mL
10x LOQ-2	5.0	100 µL of 500 ng/mL
10x LOQ-3	5.0	100 µL of 500 ng/mL
10x LOQ-4	5.0	100 µL of 500 ng/mL
10x LOQ-5	5.0	100 µL of 500 ng/mL

(**Note:** At least two additional control matrix samples were prepared to prepare matrix-matched calibration standard curve)

Step	Extraction Procedure
1	Add 10 mL of Acetonitrile (ACN) to each sample
2	Add 1 packet of 6.5g salt mixture
3	Shake the tubes by hand for about 1 minute.
4	Centrifuge tubes for 10 minutes set at 3,500 rpm at room temperature
5	**Control, LOQ and 10X LOQ samples:** Transfer 8 mL of top layer of each sample into graduated glass tubes. Evaporate contents of the glass tube close to ~1 mL in TurboVap under nitrogen set at 40 ºC. Add water to bring the final volume of sample to 2 mL.
6	Transfer an aliquot of the sample to a LC vial for LCMS/MS analysis.

The extracted samples were analyzed against matrix-matched calibration standard curve. The matrix-matched calibration standard curve was prepared by adding 50 µL of each standard into separate HPLC vials and adding 450 µL of control matrix.

Std. ID	Conc. of Source Solution (ng/mL)	Source Solution Vol. (mL)	Control Matrix Vol. (mL)	Prepared Conc. (ng/mL)
Std-8	500	0.05	0.45	50.0
Std-7	375	0.05	0.45	37.5
Std-6	250	0.05	0.45	25.0
Std-5	100	0.05	0.45	10.0
Std-4	50	0.05	0.45	5.0
Std-3	25	0.05	0.45	2.5
Std-2A	15	0.05	0.45	1.5
Std-2	10	0.05	0.45	1.0
Std-1	5	0.05	0.45	0.5

LC-MS/MS ANALYSIS

Quantitative LC-MS/MS analysis of IKF-916, CCIM, CCIM-AM, CCBA and CTCA was performed using a highly specific and sensitive MRM (Multiple Reaction Monitoring) method following HPLC separation of the analyte. The precursor ion was monitored in Q1 of the MS and the fragment ions were monitored in Q3 of the MS in the positive mode for IKF-916, CCIM and CCIM-AM. For CCBA and CTCA, Q1 and Q3 were monitored in the negative mode. The details of ions are presented in the MS/MS parameters section.

The levels of IKF-916 and its metabolites in the samples were quantified by integration of its peak area, and the concentrations were calculated by Analyst™ (version 1.6.2) by comparison with the peak area of mixed calibration standards that were analyzed in the same batch run.

LC-MS/MS System

HPLC:

Shimadzu Nexera UHPLC Component List (System X):

Component	Model
Autosampler	Sil-30acmp
Tray	Reservoir Tray
Pump	LC-30AD
Pump	LC-30AD
Column Oven	CTO-20AC
Degasser	DGU-20A5R
Controller	CBM-20A
Degasser	DGU20A5R
Tray	Reservoir Tray

Mass Spectrometer:
AB-Sciex API 6500
Analyst (Version 1.6.2)

HPLC Method

Column:	Phenomenex Synergi Hydro-RP, 4µm, 50 x 2.0 mm
Column Temperature:	30 °C
Injection volume:	5-10 µL (API6500) 25-40 µL (API4000)
Run Duration:	4 minutes

Solvent System:

Mobile Phase:	A: 5 mM Ammonium Acetate + 0.1% Formic Acid in Water B: 0.1% Formic Acid in methanol

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	% A	% B
0.5	0.8	80	20
2.5	0.8	0.0	80
3.5	0.8	0.0	80
3.6	0.8	80	20
4.2	0.8	80	20

The LC flow was diverted to the MS between 1.0 and 3.5 minutes

MS/MS Parameters for IKF-916, CCIM and CCIM-AM (Positive Mode):

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit

Ion Source Gas 1 (GS1):	30 psi
Ion Source Gas 2 (GS2):	40 psi
Curtain Gas (CUR):	20 psi
Collision Gas (CAD):	10 psi
Ion Spray Voltage (IS):	5500 V
Temperature (TEM):	500 °C
Entrance Potential (EP):	10 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	Dwell Time (msec)	CE (V)	CXP (V)
Quantitation	IKF-916	325	108	45	50	23	6.0
Confirmation	IKF-916-2	327	108	45	50	23	6.0
Quantitation	CCIM	218	183	75	50	31	12.0
Confirmation	CCIM-1	218	139	75	50	35	9.0
Quantitation	CCIM-AM	236	219	70	50	26	15.0
Confirmation	CCIM-AM-1	236	164	70	50	40	16.0

MS/MS Parameters for CCBA and CTCA (Negative Mode)

Scan Type:	MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	30 psi
Ion Source Gas 2 (GS2):	40 psi
Curtain Gas (CUR):	25 psi
Collision Gas (CAD):	10 psi
Ion Spray Voltage (IS):	-4500 V
Temperature (TEM):	500 °C
Entrance Potential (EP):	-10 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	Dwell Time (msec)	CE (V)	CXP (V)
Quantitation	CTCA	234.9	154.9	-40	100	-30	-11
Confirmation	CTCA-1	234.9	190.9	-40	100	-22	-9.0
Quantitation	CCBA	245.9	201.9	-90	100	-32	-11
Confirmation	CCBA-1	245.9	166.0	-90	100	-44	-11

METHODS OF CALCULATION

Calibration and Linearity

A series of calibration standard solutions for IKF-916 from 0.1 ng/mL to 10 ng/mL and for metabolites (CCIM, CCIM-AM, CCBA and CTCA) from 0.5 ng/mL to 50 ng/mL were injected with the validation set to quantify the residue in the samples. These calibration standards generated a linear plot of the concentration of each standard versus area with 1/x weighting. The resulting linear plot of the calibration standards had to yield a correlation coefficient (r) of at least 0.99 for the analytical set to be acceptable.

Calculation of Residue

Residues were calculated in Microsoft Excel® spreadsheets. Spreadsheets presenting the raw data are presented in Appendix D.

Representative Chromatography

Example chromatograms of IKF-916 and its metabolites standards, blank, control, LOQ and 10XLOQ samples from the method validation were presented in this report from to Figure 1 through Figure 40. The calibration curve for IKF-916 and metabolites was presented in this report from Figure 41 through Figure 50.

Statistics

The software program Microsoft Excel®, a non-validated system, generated mean, range and standard deviation of analyte recovery data. Analyst version 1.6.3 software, a non-validated system which is upgraded software for the validated Analyst version 1.6.2, was used to acquire and process data for the LC-MS/MS to calculate regression and correlation of standard curves for residue quantification.

Time Required for Analysis

A total of 8 hours was needed to prepare and analyze the set of samples by LC-MS/MS (including standard, sample preparation and analysis).