1 OBJECTIVE

The objective of this study was to develop and validate the analytical method for the determination of IKI-3106 and its metabolites NK-1375, NSY-137, TJ-537 and NU-536 in drinking water and surface water.

2 CONDUCT OF STUDY

The study was conducted at Ishihara Sangyo Kaisha, Ltd., Central Research Institute, Safety Science Research Laboratory, Environmental Sciences Group, 3-1, 2-Chome, Nishi-shibukawa Kusatsu-shi, Shiga-ken, 525-0025 Japan. The experimental start and termination dates were February 18, 2013 and May 11, 2013, respectively.

3 MATERIAL AND METHOD

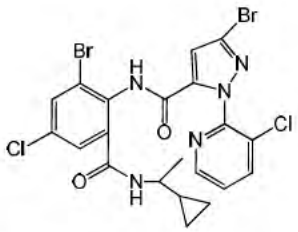
3.1 Analytical standards

3.1.1 IKI-3106

Product name: IKI-3106

Chemical name: 3-bromo-*N*-[2-bromo-4-chloro-6-[[(1-cyclopropylethyl)amino] carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide (CA)

Structure:



Molecular weight: 602.11

Lot No.: 0804

Purity: 98.8 %

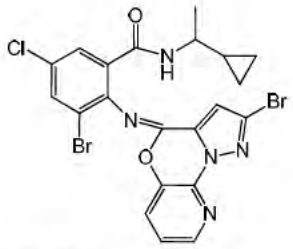
Physical state: Solid

3.1.2 NK-1375

Product name: NK-1375

Chemical name: 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-*d*]pyrido[3,2-*b*][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl) benzamide

Structure:



Molecular weight: 565.65
Lot No.: 20100825
Purity: 98.0 %
Physical state: Solid

3.1.3 NSY-137

Product name: NSY-137
Chemical name: 8-bromo-2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3-(1-cyclopropylethyl)quinazoline-4(3*H*)-one

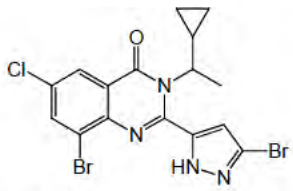
Structure:

Molecular weight: 565.65
Lot No.: 20100917
Purity: 95.9 %
Physical state: Solid

3.1.4 TJ-537

Product name: TJ-537
Chemical name: 8-bromo-2-(3-bromo-1*H*-pyrazol-5-yl)-6-chloro-3-(1-cyclopropylethyl)quinazolin-4(3*H*)-one

Structure:

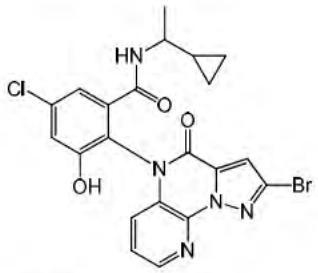


Molecular weight: 472.56
Lot No.: 20120612

Purity: 98.1 %
Physical state: Solid

3.1.5 NU-536

Product name: NU-536
Chemical name: 2-(2-bromo-4-oxopyrazolo[1,5-*a*]pyrido[3,2-*e*]pyrazin-5(4*H*)-yl)-5-chloro-*N*-(1-cyclopropylethyl)-3-hydroxybenzamide
Structure:



Molecular weight: 502.75
Lot No.: 20130321
Purity: 98.4 %
Physical state: Solid

3.2 Test water

Drinking water and surface water were used in the study. Tap water supplied by Kusatsu City was used as drinking water. Surface water was collected from Lake Biwa in Kusatsu and passed through a 0.22 μm cellulose acetate filter.

3.3 Reagents

All reagents were of analytical, HPLC or LC/MS/MS grade.

3.4 Standard solutions

3.4.1 Stock solutions

Each 10.0 mg of IKI-3106, NK-1375, NSY-137, TJ-537 and NU-536 was weighed into separate 100 mL-Volumetric flask. Acetonitrile was added to make stock standard solutions with a concentration of 100 mg/L.

3.4.2 Fortification solutions

Aliquots of each stock solution were mixed and diluted using acetonitrile:water:acetic acid (90:10:0.1, v/v/v) to make mixed fortification standard solutions with a concentration of 200 and 20 ng/mL.

3.4.3 Calibration solutions

Mixed calibration solutions, over the concentration range 0.25 to 20 ng/mL of IKI-3106, NK-1375, NSY-137, TJ-537 and NU-536 were prepared by serial dilution of the fortification solution with acetonitrile:water:acetic acid (90:10:0.1, v/v/v).

3.5 Fortification

To demonstrate the validity of the method used, untreated test water was fortified with the following levels for the IKI-3106, NK-1375, NSY-137, TJ-537 and NU-536.

0.1 ng/mL	0.5 mL of the fortification solution (20 ng/mL) was added to 100 mL of test water.
1 ng/mL	0.5 mL of the fortification solution (200 ng/mL) was added to 100 mL of test water.

3.6 Analytical method

3.6.1 Procedure

- Measure 100 mL of water sample into a 100 mL glass Erlenmeyer flask.
- Add fortification solution if required.
- Add 0.2 mL of acetic acid to the sample to acidify the solution.
- Condition an OASIS HLB Plus (225 mg) SPE cartridge, equipped with glass syringe as reservoir, with 10mL of methanol followed by 10 mL of 0.2% acetic acid in water.
- Load the sample onto the cartridge. Discard the eluate.
- Wash the cartridge with 5 mL of acetonitrile:water (20:80, v/v). Discard the eluate.
- Elute the sample with 5 mL of acetonitrile:water:acetic acid (80:20:0.2, v/v/v) followed by 5 mL of acetonitrile. Combine each eluate.
- Dilute the combined sample to volume (10 mL) with acetonitrile.
- Perform any further dilutions using acetonitrile:water:acetic acid (90:10:0.1, v/v/v), as required.
- Quantify the samples by the use of LC/MS/MS.

3.6.2 Quantitation

Quantitation of IKI-3106, NK-1375, NSY-137, TJ-537 and NU-536 was performed by LC/MS/MS using the external standard method. The calibration standards at six concentrations (0.25, 0.5, 1, 2, 10 and 20 ng/mL) were used for construction of a calibration curve. The calibration curve was constructed by plotting the peak areas against the concentration of calibration standards. From the calibration curve, the concentration of

IKI-3106, NK-1375, NSY-137, TJ-537 and NU-536 in the injected solution was determined and the residue of those analytes in the water sample was calculated.

3.7 LC/MS/MS conditions

3.7.1 HPLC

Instrument:	ACQUITY UPLC System (Waters)
Column:	BEH C18 2.1×50 mm, 1.7 μm (Waters)
Column temp.:	40℃
Mobile phase:	For IKI-3106, NSY-137, TJ-537 and NU-536 Acetonitrile:Water:Acetic acid (60:40:0.1, v/v/v) For NK-1375 Acetonitrile:Water:Acetic acid (55:45:0.1, v/v/v)
Flow rate:	0.4 mL/min
Injection volume:	2 μL
Retention time:	1.02 min (IKI-3106) 2.19 min (NK-1375) 4.09 min (NSY-137) 2.36 min (TJ-537) 0.56 min (NU-536)

3.7.2 MS/MS

Instrument:	API4000QTRAP (AB SCIEX)
Ionization mode:	ESI
Scan mode:	MRM
Mass resolution	Q1;unit, Q3;low
Heater gas temp.:	600 °C
Ion voltage:	5000 V
Gas flow settings:	Gas1;40, Gas2;70, CUR;25, CAD;11

3.7.3 Primary method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKI-3106	Pos. $[M+H]^+$	601.8	283.8	27	66	10	20
NK-1375	Pos. $[M+H]^+$	565.8	497.9	23	81	10	12
NSY-137	Pos. $[M+H]^+$	565.8	498.1	27	61	10	14
TJ-537	Pos. $[M+H]^+$	472.8	404.6	25	41	10	24
NU-536	Pos. $[M+H]^+$	503.9	418.7	45	81	10	10

3.7.4 Confirmatory method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKI-3106	Pos. $[M+H]^+$	601.8	177.0	73	66	10	28
NK-1375	Pos. $[M+H]^+$	565.8	265.8	33	81	10	44
NSY-137	Pos. $[M+H]^+$	565.8	404.9	55	61	10	10
TJ-537	Pos. $[M+H]^+$	472.8	233.3	55	41	10	34
NU-536	Pos. $[M+H]^+$	503.9	435.8	43	81	10	10