1. Introduction

The objective of this study was to validate methodology for the determination of residues of IKI-3106 and its metabolites (NK-1375, NSY-137, TJ-537 and NU-536) in drinking water and surface water, and to demonstrate a suitable confirmatory technique.

To determine the validity of the analytical method, it was necessary to determine:

- recovery / accuracy
- precision
- linearity
- specificity
- limit of detection
- limit of quantitation

This study was based upon fulfilling the requirements of the following regulatory guidelines:

Guidance for Generating and Reporting Methods of Analysis in Support of Residue Data Requirements for Annex II (part A, Section 4), and Annex III (part A, Section 5) of Directive 91/414, SANCO/3029/99 rev. 4.

SANCO/825/00 rev.8.1 of 16 November 2010 : Guidance document on residue analytical methods; to fulfil the post-registration monitoring data requirements for Annex II (part A, Section 4), and Annex III (part A, Section 5) of Council Directive 91/414/EEC as amended by Commission Directive 96/46/EC.

Commission Regulations (EU) No 283/2013 and (EU) No 284/2013 in accordance with Regulation (EC) No 1107/2009

Study organisation

The sample analysis took place at:

Huntingdon Life Sciences
Eye Research Centre
Eye
Suffolk
IP23 7PX
England

Relevant study dates

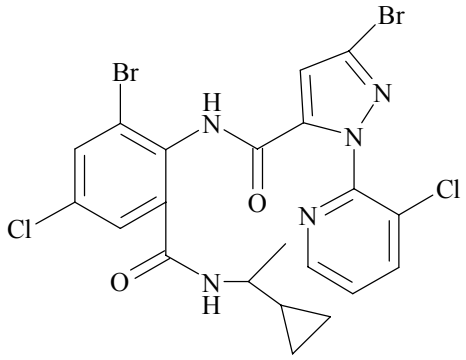
Study initiation	:	10 June 2013
Analytical phase commenced	:	10 June 2013
Analytical phase completed	:	21 June 2013

2. Materials

2.1 Analytical Standard 1 – 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)

Structural formula:

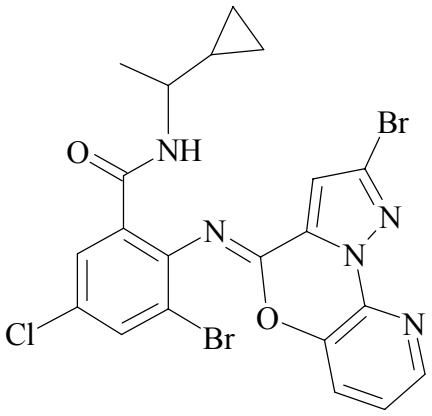


Purity:	99.18%
Expiry date:	24 January 2016
Batch number:	20110113
Physical state:	Solid
Storage conditions:	Freezer (approximately -20 ºC, in the dark)

2.2 Analytical Standard 2 – 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

Structural formula:

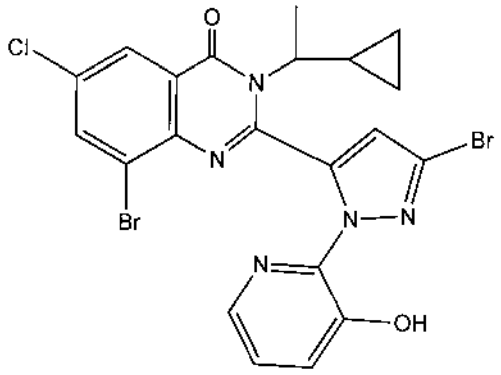


Purity:	98.60%
Expiry date:	25 July 2015
Batch number:	233-004-22-3
Physical state:	Solid
Storage conditions:	Freezer (approximately -20 ºC, in the dark)

2.3 Analytical Standard 3 – NSY-137

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

Structural formula:



Purity:	99.36% (obtained from a non-GLP study)
Expiry date:	Not available
Batch number:	314-110614-1
Physical state:	Not available
Storage conditions:	Not available

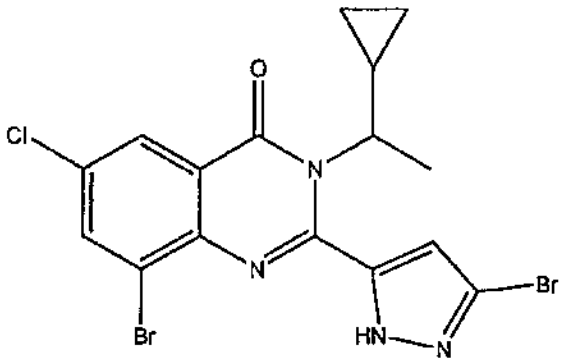
During the course of the study, following completion of the analytical work, a new Certificate of Analysis for this material became available. The updated information is presented below:

Purity:	99.3%
Expiry date:	18 July 2018
Physical state:	Solid
Storage conditions:	Freezer (approximately -20 ºC, in the dark)

2.4 Analytical Standard 4 –TJ-537

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

Structural formula:



Purity:	98.1% (obtained from a non-GLP study)
Expiry date:	Not available
Batch number:	20120612
Physical state:	Not available
Storage conditions:	Not available

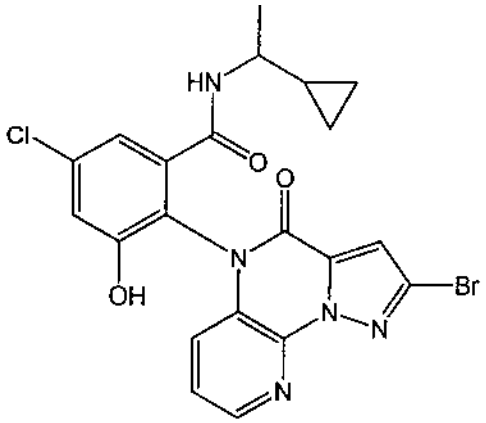
During the course of the study, following completion of the analytical work, a new Certificate of Analysis for this material became available. The updated information is presented below:

Purity:	99.1%
Expiry date:	16 July 2018
Physical state:	Solid
Storage conditions:	Freezer (approximately -20 ºC, in the dark)

2.5 Analytical Standard 5 –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

Structural formula:



Purity:	98.4% (obtained from a non-GLP study)
Expiry date:	Not available
Batch number:	20130321
Physical state:	Not available
Storage conditions:	Not available

During the course of the study, following completion of the analytical work, a new Certificate of Analysis for this material became available. The updated information is presented below:

Purity:	99.3%
Expiry date:	16 July 2018
Physical state:	Solid
Storage conditions:	Freezer (approximately -20 ºC, in the dark)

Certificates of Analysis for IKI-3106 and NK-1375, along with the new Certificates of Analysis for NSY-137, TJ-537 and NU-536, are presented in Appendix 1.

A number of standard solutions used in this study were prepared in other GLP studies being preformed for the same Sponsor. The use of these standard solutions is fully traceable to the other studies and copies of the standard preparations are included in the raw data package for the study. Some of the information provided was obtained from previous non-GLP studies, and the available relevant information is included in the study data.

2.6 Control matrices

The drinking water was obtained from a tap within the analytical laboratory. The surface water was obtained from Diss Mere, (Diss, Norfolk, England). Upon receipt the surface water sample was allocated a unique Huntingdon Life Sciences, Environmental Analysis Department identification number. The water was characterised in a separate study and the characterisation data is presented in the following table:

Parameter	Found value (surface water)
pH	8.10
Conductivity	392 µS/cm
Alkalinity	204 mg/l as $CaCO_3$
Total Hardness	199 mg/l as $CaCO_3$
Total Organic Carbon	12.871 mgC/L
Dissolved Organic Carbon	11.549 mgC/L

3. Methods

3.1 Validation

Sub-samples of each of the two water types were fortified with known concentrations of the five test substances and analysed according to the following regime:

2 sub-samples of untreated sample water
5 sub-samples of untreated sample water fortified at the LOQ (0.1 µg/L)
5 sub-samples of untreated sample water fortified at 1 µg/L

These samples were then analysed using the analytical methodology, with each sample injected onto the chromatographic system once.

3.2 Final extract stability

An experiment was set up to demonstrate the stability of the five analytes under the typical storage conditions of the final extracts if they are not quantified immediately after preparation. Processed control extracts, fortified with all five analytes were stored at approximately -20 ºC in the dark (i.e. in a freezer).

Aliquots of each of the control sample extracts were fortified with the five analytes at a concentration of 10 ng analyte/mL of final extract. The concentration of analytes in the stored extracts was determined at day 0 and after 7 days. The concentration of the analytes in freshly fortified control extracts was also determined at that time.

3.3 Matrix effects

Any possible sample matrix effects were investigated by the comparison of the instrument response to the analytes in the fortified final extract samples with the response of the analytes in solvent based calibration standard solutions prepared at the same time.

3.4 Analytical method

Samples were acidified prior to extraction/concentration using solid phase extraction (SPE). Quantitation was performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

The analytical method used in the laboratory is presented in Appendix 3.

3.5 Fortification/calibration solutions

Individual stock standard solutions of the five analytes were prepared by dissolving an accurately weighed amount of each material in a suitable volume of acetonitrile. These stock solutions were further diluted with acetonitrile to produce mixed fortification solutions at 10 µg/mL, 1 µg/mL and 0.1 µg/mL concentrations. Appropriate corrections were made for the purity of each material.

The instrument calibration solutions, over the concentration range 0.25 ng/mL to 20 ng/mL, were prepared by serial dilution of the mixed fortification solution in acetonitrile:water:acetic acid (90:10:0.1, v:v:v), as detailed below:

Standard solution used (ng/mL)	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
100	2	10	20
100	1.5	10	15
100	1	10	10
100	0.5	10	5
100	0.25	10	2.5
10	1	10	1
10	0.5	10	0.5
10	0.25	10	0.25

3.6 Calculation of results for validation samples

Test samples were quantified using the following equation:

$$\text{Residue found (µg/L)} = x \times \frac{1}{\text{M}} \times \text{D}$$

Where x (residue concentration in final solution) was calculated using the linear regression

$$y = \text{m}\,x + \text{c} \qquad \text{where } x \text{ (concentration in ng/mL)} = \frac{y - \text{c}}{\text{m}}$$

c	=	intercept
m	=	slope
y	=	peak area of sample
M	=	matrix concentration (mL/mL)
D	=	dilution factor

Example calculation of IKI-3106 detected in surface water fortified at 0.1 µg/L (analytical identification 13/00/1482 F0.1 A, analysis batch 2). The primary data for this sample is presented in Table 41, Appendix 2.

Linear regression $y = \text{m}\,x + \text{c}$

$$8.08920\text{e}3 = 9966.76x + 0.0390055$$

where
$y = 8.08920\text{e}3$
$\text{m} = 9966.76$
$\text{c} = 0.0390055$

$$\text{Therefore, concentration of IKI-3106 } (x) = \frac{8.08920\text{e}3 - 0.0390055}{9966.76} = 0.8116 \text{ ng/mL}$$

Matrix concentration = 10 mL matrix/mL final extract
Dilution factor = 1

$$\text{IKI-3106 detected (µg/L)} = \frac{0.8116 \text{ ng/mL} \times 1}{10 \text{ mL/mL}} = 0.08116 \text{ µg/L}$$

$$\text{Recovery (\%)} = \frac{0.08116 \text{ µg/L} \times 100}{0.1 \text{ µg/L}} = 81\%$$

Appendix 3 Analytical Method

DETERMINATION OF IKI-3106, NK-1375, NU-536, TJ-537 AND NSY-137 IN WATER

1. General principle

Samples are acidified with acetic acid prior to extraction with Oasis HLB Plus SPE cartridges. Quantitation is performed using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

2. Apparatus, glassware etc

Balances (various ranges)
Volumetric flasks (various sizes)
Syringes (various sizes)
Volumetric pipettes (various sizes)
Polypropylene tubes (15 mL)
Polyethylene bottles (250 mL)
Measuring cylinders (various sizes)
Solid phase extraction vacuum manifold

3. Materials	**Typical Grade (or equivalent)**
Acetonitrile	HPLC
Acetic acid	AR
Water	HPLC
Oasis HLB Plus cartridges (225 mg)	

4. Preparation of reagents

Preparation of acetonitrile:water (20:80 v:v) - acetonitrile (200 mL) is mixed thoroughly with water (800 mL).

Preparation of acetonitrile:water:acetic acid (90:10:0.1 v:v:v) - acetonitrile (900 mL) is mixed thoroughly with water (100 mL) and acetic acid (1 mL).

Preparation of 0.2% acetic acid in water (v:v) – acetic acid (2 mL) is mixed thoroughly with water (1 L).

Preparation of water:acetonitrile:acetic acid (90:10:0.1 v:v:v) - acetonitrile (100 ml) and acetic acid (1 ml) is added to HPLC water (900 ml) and mixed thoroughly prior to use.

Preparation of acetonitrile: acetic acid (100:0.1 v:v) - acetonitrile (1000 mL) is mixed thoroughly with acetic acid (1 mL).

5. Analytical standard solutions

An appropriate amount of each test substance is accurately weighed and dissolved in acetonitrile to give the individual stock standard solutions. Appropriate dilutions of the stock standard solutions are made with acetonitrile to give mixed fortification standard solutions (e.g. 1 µg/mL and 0.1 µg/mL). Appropriate corrections are made for the purity of each test substance.

The mixed fortification solutions are progressively diluted with acetonitrile:water:acetic acid (90:10:0.1 v:v:v) to produce a series of instrument calibration solutions in the range 0.25 to 20 ng/mL.

6. Procedure

6.1 Measure the water sample (100 mL) into a 250 mL polyethylene bottle.
6.2 Add fortification solution at this stage if required.
6.3 Add acetic acid (0.2 mL) and acetonitrile (10 mL), and mix well.
6.4 Condition the Oasis HLB Plus SPE cartridge with acetonitrile (10 mL) and 0.2% acetic acid in water (10 mL), discarding the eluate.
6.5 Load the sample from step 6.3 onto the SPE cartridge, discarding the eluate.
6.6 Wash the cartridge with an aliquot (5 mL) of acetonitrile:water (20:80 v:v), discarding the eluate.
6.7 Elute the SPE cartridge with an aliquot (10 mL) of acetonitrile:water:acetic acid (90:10:0.1, v:v:v) collecting in a 15 mL polypropylene tube.
6.8 Dilute the final extract to volume (10 mL) with acetonitrile. Final matrix concentration ≡ 10 mL matrix / mL final solution.
6.9 Perform any further dilutions using acetonitrile:water:acetic acid (90:10:0.1, v:v:v), as required.
6.10 Quantify the samples by the use of LC-MS/MS.

7. Flow chart of analytical procedure

Measure 100 mL sub-sample into polyethylene bottle
↓
Fortification with analytical standards (if required)
↓
Add acetic acid and acetonitrile
↓
Extraction/clean-up by OASIS HLB
↓
Dilute to volume with acetonitrile
↓
Quantitative analysis by LC-MS/MS

8. LC-MS/MS conditions

Instrument:	AB Sciex API 4000 (Analyst 1.4.2 software) coupled to Waters Acquity UPLC system
Mode:	Ionspray positive
Ion monitoring details:	IKI-3106: *m/z* 602>284 IKI-3106: *m/z* 602>177 (confirmatory) NK-1375: *m/z* 566>498 NK-1375: *m/z* 566>266 (confirmatory) NU-536: *m/z* 504>419 NU-536: *m/z* 504>436 (confirmatory) TJ-537: *m/z* 473>405 TJ-537: *m/z* 473>233 (confirmatory) NSY-137: *m/z* 566>498 NSY-137: *m/z* 566>405 (confirmatory)
Column:	Acquity UPLC® BEH C_{18} (2.1 mm x 50 mm, 1.7 µm), or equivalent, column temperature 40°C
Mobile phase A:	Water:acetonitrile:acetic acid (90:10:0.1 v:v:v)
Mobile phase B:	Acetonitrile: acetic acid (100:0.1 v:v)
Gradient:	Isocratic 35% A, 65% B
Cycle time:	4 min
Injection volume:	10 µL
Flow rate:	0.4 mL/min
Retention time:	IKI-3106 - approximately 0.7 minutes NK-1375 – approximately 0.9 minutes NU-536 - approximately 0.5 minutes TJ-537 – approximately 1.2 minutes NSY-137 – approximately 1.8 minutes
LOQ: LOD:	0.1 µg/L 0.25 ng/mL (≡ 0.025 µg/L in sample matrix)

NOTE – alternative instruments may also be used, operated under conditions that are considered to be equivalent to those described above. However, some differences may be observed in the resulting data, such as slight differences in analyte retention times, or the observed sensitivity of the ion transitions monitored.