1. Introduction

The objective of this study was to validate methodology for the determination of residues of folpet and five of its metabolites (phthalimide, phthalic acid, phthalamic acid, 2-cyanobenzoic acid and benzamide) in drinking water.

The protocol was signed by the Study Director and Huntingdon Life Sciences Management on 18 November 2010 and by the Sponsor on 21 November 2010.

The study was undertaken at Huntingdon Life Sciences, Eye between 18 February 2011 and 21 March 2011.

The study was designed and performed in accordance with 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.7 of 17 March 2004

The signed protocol, a copy of the final report and the primary data pertaining to the study has been retained in the archives of Huntingdon Life Sciences.

2. Materials

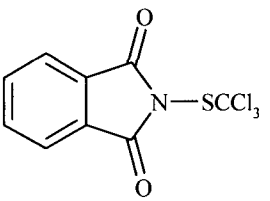
2.1 Analytical Standard – Folpet

Common names: Folpet, Folpan

Chemical name (IUPAC): N-(trichloromethylthio)phthalimide

CAS registry number: 133-07-3

Structure:



Molecular formula: $C_9H_4Cl_3NO_2S$

Molecular weight: 296.6

Storage conditions: Ambient, desiccate

Supplier: Sponsor

Lot number: 381-101-01

Purity: 99.3%

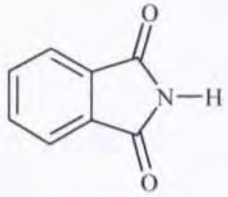
Receipt date: 26 November 2010

Expiry date: 19 November 2011

Appearance: White crystalline solid

A certificate of analysis is presented in Annex 1.

2.2 Analytical Standard – Phthalimide

Common names:	Phthalimide
Chemical Abstracts name :	1,3-Dihydro-1,3-dioxoisoindole
CAS registry number:	85-41-6
Structure:	
Molecular formula:	$C_8H_5NO_2$
Molecular weight:	147.1
Storage conditions:	Ambient, desiccate
Supplier:	Sponsor
Lot number:	427-027-00
Purity:	99%
Receipt date:	26 November 2010
Expiry date:	10 October 2013
Appearance:	White crystalline solid

A certificate of analysis is presented in Annex 1.

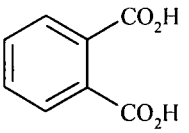
2.3 Analytical Standard – Phthalic acid

Common names: Phthalic acid

Chemical Abstracts name: 1,2-Benzenedicarboxylic acid

CAS registry number: 88-99-3

Structure:



Molecular formula: $C_8H_6O_4$

Molecular weight: 166.1

Storage conditions: Ambient, desiccate

Supplier: Sponsor

Lot number: 326-013-00

Purity: 99.5%

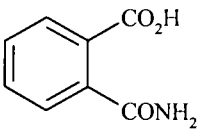
Receipt date: 26 November 2010

Expiry date: 27 November 2011

Appearance: White crystalline solid

A certificate of analysis is presented in Annex 1.

2.4 Analytical Standard – Phthalamic acid

Common names:	Phthalamic acid
Chemical Abstracts name:	2-(aminocarbonyl)-benzoic acid
CAS registry number:	88-97-1
Structure:	
Molecular formula:	$C_8H_7NO_3$
Molecular weight:	165.1
Storage conditions:	Ambient, desiccate
Supplier:	Sponsor
Lot number:	326-014-00
Purity:	97%
Receipt date:	26 November 2010
Expiry date:	22 October 2012
Appearance:	White crystalline solid

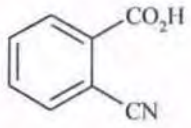
A certificate of analysis is presented in Annex 1.

2.5 Analytical Standard – 2-Cyanobenzoic acid

Common names: 2-Cyanobenzoic acid

CAS registry number: 3839-22-3

Structure:



Molecular formula: C_8H_5NO2

Molecular weight: 147.1

Storage conditions: Ambient

Supplier: Sigma-Aldrich

Lot number: 02530EJ

Purity: 94.7%

Receipt date: 16 November 2010

Expiry date: 16 November 2011

Appearance: White powder

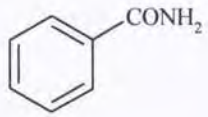
A certificate of analysis is presented in Annex 1.

2.6 Analytical Standard – Benzamide

Common names: Benzamide

CAS registry number: 55-21-0

Structure:



Molecular formula: C_7H_7NO

Molecular weight: 121.1

Storage conditions: Ambient

Supplier: Sigma-Aldrich

Lot number: STBB1249

Purity: >99.9%

Receipt date: 16 November 2010

Expiry date: 16 November 2011

Appearance: Off-white crystalline powder

A certificate of analysis is presented in Annex 1.

2.7 Control matrix

Untreated drinking water used for the validation of the analytical method was obtained from the local supply as required. Upon receipt a unique Huntingdon Life Sciences Environmental Analysis Identification Number was assigned.

3. Experimental

3.1 Validation

Sub-samples of drinking water were fortified with known concentrations of folpet, phthalimide, 2-cyanobenzoic acid and benzamide and analysed according to the following regime:

2 sub-samples of untreated drinking water
5 sub-samples of untreated drinking water fortified at the LOQ (0.1 µg/L)
5 sub-samples of untreated drinking water fortified at 1.0 µg/L

Sub-samples of drinking water were fortified with known concentrations of phthalic acid and phthalamic acid and analysed according to the following regime:

2 sub-samples of untreated drinking water
5 sub-samples of untreated drinking water fortified at the LOQ (2.0 µg/L)
5 sub-samples of untreated drinking water fortified at 20 µg/L

3.2 Analytical methodology

For folpet the method comprised of extraction by liquid:liquid partition with toluene. For phthalimide the method comprised of extraction by liquid:liquid partition with dichloromethane. For both folpet and phthalimide quantitation was performed using gas chromatography with mass spectrometric detection (GC-MS).

Phthalic acid was quantified directly using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

For phthalamic acid and benzamide the method comprised of acidification followed by quantitation by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS)

For 2-cyanobenzoic acid the method comprised of solid phase extraction (SPE) using NH_2 cartridges. Quantitation was performed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS)

The analytical method is detailed in Annex 2 of this report.

3.3 Final extract stability

Aliquots of untreated drinking water final extract were fortified with folpet, phthalimide, phthalic acid, phthalamic acid, 2-cyanobenzoic acid and benzamide at concentrations within the calibration range. These were analysed immediately (Day 0) and after 7 days freezer storage (nominally -20°C). A freshly fortified extract of drinking water was also prepared and analysed on Day 7 to act as a procedural recovery.

3.4 Calculation of results for validation samples

Calibration curves were prepared on the basis of peak area of the analyte versus concentration of that analyte in the calibration standard, applying a least squares linear regression fit. Test samples were quantified using the following equation:

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

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

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

c	=	intercept
m	=	slope
y	=	peak area of sample
M	=	matrix concentration in final extract (mL/mL)
D	=	dilution factor – any further dilution required on final extract

Example calculation of folpet detected in drinking water fortified at 1.0 µg/L (11/00/1212 F.1.0 A – quantitation method). This sample is presented in the summary Table 1.

Linear regression $y = m\,x + c$

$$6909.147 = 1869.54x - 109.471$$

where $y = 6909.147$
$m = 1869.54$
$c = -109.471$

Therefore, concentration of folpet in final solution (x)

$$= \frac{6909.147 + 109.471}{1869.54} = 3.754 \text{ ng/mL}$$

Matrix concentration = 4 mL/mL (40 mL → 10 mL)
Dilution factor = 1

$$\text{Folpet detected } (\mu g/L) = \frac{3.754 \text{ ng/mL} \times 1}{4 \text{ mL/mL}} = 0.939 \text{ µg/L}$$

$$\text{Recovery} = \frac{0.939 \text{ µg/L} \times 100\%}{1.0 \text{ µg/L}} = 94\%$$

Annex 2 Analytical Method

1. General principles

Folpet - Water samples were extracted by liquid:liquid partitioning with toluene, followed by quantitation by gas chromatography with mass spectrometric detection (GC-MS).

Phthalimide - Water samples were extracted by liquid:liquid partitioning with dichloromethane, followed by quantitation by gas chromatography with mass spectrometric detection (GC-MS).

Phthalic acid - Water samples were quantified directly by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

Phthalamic acid - Water samples were acidified followed by quantitation by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

2-Cyanobenzoic acid – Water samples were acidified then extracted using NH_2 solid phase extraction (SPE) cartridges. Quantitation was by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

Benzamide - Water samples were acidified followed by quantitation by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

2. Materials

Acetonitrile	HPLC
Ammonia (S.G. 0.88)	Analytical Reagent
Ammonium formate	Analytical Reagent
Dichloromethane	HPLC
Diglyme (bis(2-methoxyethyl) ether)	Not specified
Formic acid	HPLC
Methanol	HPLC
NH_2 solid phase extraction cartridges	500 mg, 3 mL (Phenomenex)
Orthophosphoric acid (S.G. 1.7)	HPLC
Sodium chloride	Lab Reagent
Sodium Sulphate (anhydrous)	Analytical Reagent
Toluene	Glass distilled
Water	HPLC

3. Preparation of solvents

Formic acid (1 M):

Add formic acid (38 mL) to water (1000 mL) in a suitable bottle, cap and shake thoroughly to mix.

Formic acid (0.01 M):

Add formic acid (10 mL, 1 M) to water (990 mL) in a suitable bottle, cap and shake thoroughly to mix.

2% diglyme in dichloromethane:

Add diglyme (20 mL) to dichloromethane (980 mL) in a suitable bottle, cap and shake thoroughly to mix.

2% diglyme in toluene:

Add diglyme (20 mL) to toluene (980 mL) in a suitable bottle, cap and shake thoroughly to mix.

Orthophosphoric acid (2 N):

Add orthophosphoric acid (4.4 mL) to water (95.6 mL) in a suitable bottle, cap and shake thoroughly to mix.

Ammonium Formate (0.01 M) in methanol:water (20:80 v:v):

Dissolve ammonium formate (0.6g) in water (800 mL) in a suitable bottle. Add methanol (200 mL), cap the bottle and shake thoroughly to mix.

10% ammonia in methanol:

Add ammonia solution (20 mL) to methanol (180 mL) in a suitable bottle, cap and shake thoroughly to mix.

Mobile phase A

Dissolve ammonium formate (0.6 g) in water (900 mL) in a suitable bottle. Add methanol (100 mL) and formic acid (1 mL), cap the bottle and shake thoroughly to mix.

Mobile phase B

Add formic acid (1 mL) to methanol (1000 mL) in a suitable bottle, cap and shake thoroughly to mix.

4. Test substances stock and fortifying solutions

Appropriate amounts of the analytical standards (corrected for purity) were accurately weighed and dissolved in appropriate solvents to give stock standard solutions. Appropriate dilutions of the stock standard solutions were made with acetonitrile to give a series of fortification standard solutions.

Folpet - The fortification solutions were progressively diluted with 2% diglyme in toluene to produce a series of instrument calibration solutions in the range 0.2 to 10 ng/mL.

Phthalimide - The fortification solutions were progressively diluted with 2% diglyme in dichloromethane to produce a series of instrument calibration solutions in the range 0.2 to 10 ng/mL.

Phthalic acid - The fortification solutions were progressively diluted with water to produce a series of instrument calibration solutions in the range 1 to 50 ng/mL.

Phthalamic acid - The fortification solutions were progressively diluted with 0.01M formic acid to produce a series of instrument calibration solutions in the range 1 to 50 ng/mL.

2-Cyanobenzoic acid - The fortification solutions were progressively diluted with 0.01 M ammonium formate in methanol:water (20:80 v:v) to produce a series of instrument calibration solutions in the range 1 to 50 ng/mL.

Benzamide - The fortification solutions were progressively diluted with 0.01M formic acid to produce a series of instrument calibration solutions in the range 0.05 to 2 ng/mL.

5. Procedure

Procedure - Folpet

1. Transfer drinking water (40 mL) to a polypropylene tube (50 mL).
2. Fortify as required.
3. Add sodium chloride (~5 g), Orthophosphoric acid (1 mL, 2 N) and toluene (5 mL) to the sample, cap and shake vigorously by hand for approximately 1 minute.
4. Allow the phases to separate and transfer the upper organic phase to a scintillation vial containing anhydrous sodium sulphate (~2 g).
5. Add a further aliquot of toluene (5 mL) to the remaining aqueous phase and shake vigorously by hand for approximately 1 minute.
6. Allow the phases to separate and transfer the upper organic phase to the scintillation vial containing the first toluene extract.
7. Transfer the combined extracts to a graduated polypropylene tube (15 mL).
8. Add diglyme (200µL) and make to volume (10 mL) by the addition of toluene prior to quantification of folpet by GC-MS.

Procedure - Phthalimide
(Note - avoid plastic wherever possible for the determination of phthalimide)

1. Transfer drinking water (40 mL) to a glass tube (50 mL).
2. Fortify as required.
3. Add dichloromethane (5 mL) to the sample, cap and shake vigorously by hand for approximately 1 minute.
4. Allow the phases to separate and transfer the upper organic phase to a scintillation vial containing anhydrous sodium sulphate (~2 g).
5. Add a further aliquot of dichloromethane (5 mL) to the remaining aqueous phase and shake vigorously by hand for approximately 1 minute.
6. Allow the phases to separate and transfer the upper organic phase to the scintillation vial containing the first dichloromethane extract.
7. Transfer the combined extracts to a volumetric flask (10 mL).
8. Add diglyme (200µL) and make to volume (10 mL) by the addition of dichloromethane prior to quantification of phthalimide by GC-MS.

Procedure – Phthalic acid
(Note - avoid plastic wherever possible for the determination of phthalic acid)

1. Transfer drinking water (10 mL) to a glass vial (20 mL).
2. Fortify as required.
3. Shake to mix prior to quantification of phthalic acid by LC-MS/MS.

Procedure – Phthalamic acid
(Note - avoid plastic wherever possible for the determination of phthalamic acid)

1. Transfer drinking water (10 mL) to a glass vial (20 mL).
2. Fortify as required.
3. Add formic acid (100 µL, 1 M) to the sample, cap and shake to mix prior to quantification of phthalamic acid by LC-MS/MS.

Procedure – 2-Cyanobenzoic acid

1. Transfer drinking water (40 mL) to a polypropylene tube (50 mL).
2. Fortify as required.
3. Add formic acid (0.4 mL, 1 M).
4. Condition a NH_2 SPE cartridge with methanol (3 mL) and 0.01 M formic acid (3 mL).
5. Pass the acidified sample from step 3 through the cartridge and discard the eluate.
6. Pull the SPE cartridge dry for 10 minutes.
7. Elute the cartridge with 10% ammonia in methanol (6 mL) and collect the eluate in a scintillation vial.
8. Transfer to a round bottom flask (100 mL) and rotary evaporate at approximately 40°C until dry.
9. Add 0.01M ammonium formate in methanol:water (20:80 v:v, 2 mL) and reconstitute with the aid of ultrasonication prior to quantification of 2-cyanobenzoic acid by LC-MS/MS.

Procedure – Benzamide

1. Transfer drinking water (10 mL) to a glass vial (20 mL).
2. Fortify as required.
3. Add formic acid (100 µL, 1 M) to the sample, cap and shake to mix prior to quantification of benzamide by LC-MS/MS.

6. Chromatographic analysis

GC-MS – Quantitation conditions (Folpet and Phthalimide)

Instrument:	Hewlett Packard 5890 Gas Chromatograph with a Trio 1000 mass spectrometric detector (GC-MS)
Ionisation mode:	Negative chemical ionisation (CI -)
Ion monitoring details:	Folpet *m/z* 146 Phthalimide *m/z* 147
Column:	Optima-17 or equivalent (30 m x 0.25 mm x 0.5µm film thickness)
Temperature programme:	150°C held for 2 minutes then ramped at 15°C/minute to 300°C, held for 6.5 minutes
Injector temperature:	200°C
Transfer line:	260°C
Injection volume:	5 µL (Splitless)
Gas flow rate:	Carrier (Helium) at 1 mL/min
Retention time:	Folpet Approximately 13.8 minutes Phthalimide Approximately 8.3 minutes
LOD:	0.2 ng/mL (equivalent to 0.05 µg/L in water)
LOQ:	0.1 µg/L in water

GC-MS – Confirmatory conditions (Folpet and Phthalimide)

Instrument:	Hewlett Packard 5890 Gas Chromatograph with a Trio 1000 mass spectrometric detector (GC-MS)
Ionisation mode:	Negative chemical ionisation (CI -)
Ion monitoring details:	Folpet *m/z* 146 Phthalimide *m/z* 147
Column:	DB-5 or equivalent (30 m x 0.25 mm x 0.25µm film thickness)
Temperature programme:	
Folpet	150°C held for 2 minutes then ramped at 15°C/minute to 300°C, held for 6.5 minutes
Phthalimide	100°C held for 2 minutes then ramped at 7.5°C/minute to 220°C then ramped at 50°C/minute to 300°C, held for 4 minutes
Injector temperature:	200°C
Transfer line:	260°C
Injection volume:	5 µL (Splitless)
Gas flow rate:	Carrier (Helium) at 1 mL/min
Retention time:	Folpet Approximately 9.6 minutes Phthalimide Approximately 10.5 minutes
LOD:	0.2 ng/mL (equivalent to 0.05 µg/L in water)
LOQ:	0.1 µg/L in water

LC-MS conditions (2-Cyanobenzoic acid and Benzamide)

Instrument:	AB Sciex API4000
Ionisation mode:	Benzamide - Positive ionspray 2-cyanobenzoic acid – Negative ionspray
Ion monitoring details:	
Benzamide	MRM *m/z* 122>105 (Quantitation) MRM *m/z* 122>77 (Confirmation ion)
2-cyanobenzoic acid	MRM *m/z* 146>102 (Quantitation) MRM *m/z* 192>146 (Confirmation ion)
Column:	Acquity UPLC BEH C_{18} 1.7 µm (50 × 2.1 mm, Waters)
Column temperature:	45°C
Mobile phase A:	Water:methanol:formic acid (90:10:0.1 v:v:v) containing 0.01 M ammonium formate
Mobile phase B:	Methanol containing 0.1% formic acid

Gradient:

Time (min)	%A	%B
0	100	0
0.2	100	0
2	0	100
2.5	0	100
3	100	0
4	100	0

Flow rate:	0.5 mL/min
Injection volume:	10 µL
Retention time:	
Benzamide	approx. 1 minute
2-Cyanobenzoic acid	approx. 1 minute
LOQ:	0.1 µg/L in water
LOD:	
Benzamide	0.05 ng/mL (equivalent to 0.05 µg/L in water)
2-Cyanobenzoic acid	1 ng/mL (equivalent to 0.05 µg/L in water)

LC-MS conditions (Phthalic acid and Phthalamic acid)

Instrument:	Waters TQD
Ionisation mode:	Phthalamic acid- Positive electrospray Phthalic acid – Negative electrospray
Ion monitoring details:	
Phthalamic acid	MRM *m/z* 166>149 (Quantitation) MRM *m/z* 166>121 (Confirmation ion)
Phthalic acid	MRM *m/z* 165>77 (Quantitation) MRM *m/z* 165>121 (Confirmation ion)
Column:	Acquity UPLC BEH C_{18} 1.7 µm (50 × 2.1 mm, Waters)
Column temperature:	45°C
Mobile phase A:	Water:methanol:formic acid (90:10:0.1 v:v:v) containing 0.01 M ammonium formate
Mobile phase B:	Methanol containing 0.1% formic acid

Gradient:

Time (min)	%A	%B
0	100	0
0.2	100	0
2	5	95
2.5	5	95
3	100	0
4	100	0

Flow rate:	0.5 mL/min
Injection volume:	10 µL
Retention time:	
Phthalamic acid	approx. 1 minute
Phthalic acid	approx. 0.6 minute
LOQ:	2 µg/L in water
LOD:	
Phthalamic acid	1 ng/mL (equivalent to 1 µg/L in water)
Phthalic acid	1 ng/mL (equivalent to 1 µg/L in water)