INTRODUCTION

An independent method validation study was conducted at PTRL West, Inc. (625-B Alfred Nobel Dr, Hercules, California) to determine the validity of a procedure to analyze Bensulide in River Water. See Appendix A for the study Protocol. The study was initiated on November, 21, 2011. The independent laboratory validation was conducted from November 22 2011 through January 23 2011.

2.0 MATERIALS AND METHODS

2.1 Method

The sample preparation (SPE cleanup) section of the analytical method for the analysis of Bensulide in water was conducted relative to a method validated at ABC Laboratories (Reference 1, See Also Appendix A- Protocol Appendix 1).

The determination of Bensulide was validated by spiking known concentrations of analyte into control water samples. Samples were applied to a pre-conditioned SPE cartridge. The analytes were eluted with methanol. The eluent was then evaporated to dryness and then resuspended with acetonitrile/water (70:30, v/v) and submitted for LC/MS analyses in the positive mode. The percent recovery was determined relative to an external calibration curve.

2.2 Test System

The test system was river water. The control water sample used for this study had an identification number of 2111W-064 and was received from Agvise Laboratories, Inc. on November 17, 2011. The water sample originated from North Dakota (Agvise site name : Goose River, Grand Forks County, ND).

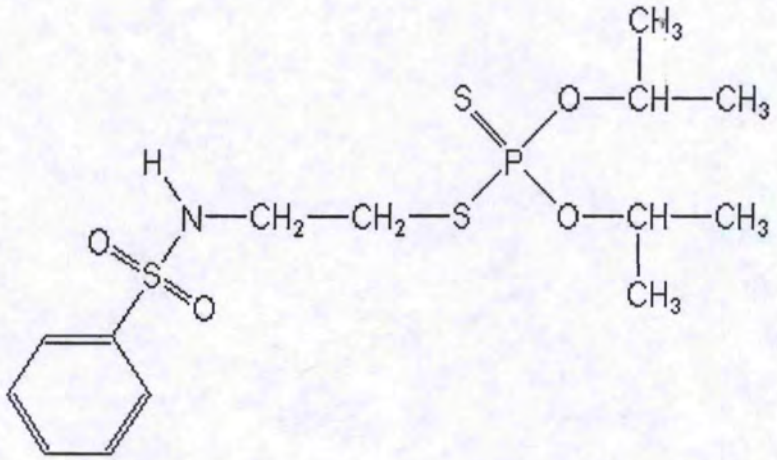
2.3 Reference Substances

The Bensulide (2228W-003) reference substance was obtained from Gowan Company. Stock solutions of the reference substance were prepared at 1.06 and 0.97 mg/mL in acetonitrile and were taken to be stable over the period of the study. Calibrant solutions appeared stable when stored refrigerated for at least 2 days, based on the comparison of LC-MS/MS chromatograms during this study. The certificate of analysis for the reference substance is provided in Appendix B.

Compound: **Bensulide**

IUPAC Name: O, O-diisopropyl S-2-phenylsulfonylaminoethyl phosphorodithioate

Chemical Structure:



Purity: 97.7%

Lot Number: TQ-BTS-1105-050

PTRL West Inventory Number: 2228W-003

Date Received: August 18, 2011

Expiration Date: February 25, 2013

Storage Conditions: Room Temperature

2.4 Equipment

Amber glass vials, 7 mL

Balance (various types)

Flat bottom flasks

Graduated cylinder, various sizes

Pasteur pipettes

Polypropylene centrifuge tubes 15 mL

SPE cartridges. J.T. Baker BAKERBOND(C_{18}, 6 mL, 500 mg))

Syringes, microliter, various sizes

Vacuum evaporator, (Zymark TurboVap LV evaporator)

Vacuum manifold with stopcocks for SPE cartridges

Volumetric flask, various sizes

Vacuum pump
Wrist-action shaker

2.5 Solvents and Reagents

All solvents and reagents (reagent grade or better) were obtained from Fisher Scientific or VWR.

Acetonitrile
Formic Acid
Methanol
Water

2.6 Reference Substance Stock Solution Preparation

Stock standard solutions of Bensulide (corrected for purity) were prepared at 1.06 and 0.97 mg/mL in acetonitrile. The stock standards were stored refrigerated (at approximately 4°C) when not in use.

2.7 Preparation of Intermediate/Fortification Solutions

Individual solutions were prepared as follows:

106.0 µg/mL Intermediate solution A:

1 ml of the 1.06 mg/mL stock solution was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

10.6 µg/mL Intermediate solution A:

1 ml of the 106 µg/mL Intermediate solution A was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

1.06 µg/mL Intermediate solution A:

1 ml of the 10.6 µg/mL Intermediate solution A was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

97 µg/mL Intermediate solution B:

1 ml of the 0.97 mg/mL stock solution was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

9.7 µg/mL Intermediate solution B:

1 ml of the 97 µg/mL Intermediate solution A was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

0.97 µg/mL Intermediate solution B:

1 ml of the 97 µg/mL Intermediate solution A was transferred into individual 10 ml volumetric flasks. The stock solution was diluted to the mark with acetonitrile:water (70:30) and mixed well.

Intermediate/Fortification solutions were transferred to 7 ml amber glass vials and stored in a refrigerator when not in use.

2.8 Preparation of Calibrants

Calibration standard solutions were prepared as follows:

- Calibrant # 1, 106 ng/mL: 1000 µL of the 1.06 µg/mL intermediate solution A was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.
- Calibrant # 2, 72.75 ng/mL: 750 µL of the 0.97 µg/mL intermediate solution B was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.
- Calibrant # 3, 53 ng/mL: 500 µL of the 1.06 µg/mL intermediate solution A was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.
- Calibrant # 4, 24.25 ng/mL: 250 µL of the 0.97 µg/mL intermediate solution B was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.

- Calibrant # 5, 10.6 ng/mL: 1000 µL of the 106 ng/mL calibrant # 1 standard solution was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.
- Calibrant # 6, 4.85 ng/mL: 2 mL of the 24.25 ng/mL calibrant # 4 solution was transferred into a 10 ml volumetric flask. Diluted to the mark with acetonitrile:water (70:30) and mixed well.

Standards were mixed well in volumetric flasks, then transferred to 7 mL amber glass vials. All calibration solutions were stored refrigerated when not in use.

2.9 Sample Preparation

The control water sample was received from Agvise Laboratories, Inc. on November 17, 2011. The water sample originated from North Dakota (Agvise site name : Goose River, Grand Forks County, ND).

Water samples (200 mL, 0.2 kg) were aliquoted into flat bottom flasks prior to fortification (if necessary) and extraction.

2.10 Sample Fortification

Fortification of control water was performed to analyze method percent recoveries for ILV. A portion (200 mL of water) was fortified with Fortification solution as follows:

Fortification Designation	Fortification Level (µg/kg)	Concentration of Fortification solution used
F1A	1.0	1.0 µg/mL
F1B	1.0	1.0 µg/mL
F1C	1.0	1.0 µg/mL
F1D	1.0	1.0 µg/mL
F1E	1.0	1.0 µg/mL
F2A	200	100 µg/mL
F2B	200	100 µg/mL
F2C	200	100 µg/mL
F2D	200	100 µg/mL
F2E	200	100 µg/mL

2.11 Extraction Method

1 Using a graduated cylinder, measure sample volumes to 200 mL. Transfer to flat bottom flasks.

2 Fortify samples as necessary.

3 Using a vacuum system, condition J.T. Baker BAKERBOND SPE cartridges (C_{18}, 6 mL, 500 mg) with two 5 mL aliquots of HPLC methanol followed by two 5 mL aliquots of DI water at a flow rate of 2-3 drops per second. Do not allow the cartridges to go dry.

4 Apply samples to the cartridges using reservoir adapters. Discard the eluents. Residues are retained on the cartridge.

5 Rinse flasks twice with 10 mL of DI water and apply to the cartridges. Dry cartridges for ~5 min. after the entire sample have been applied. Discard eluents.

6 Place a 15 mL polypropylene centrifuge tube under the SPE cartridge.

7 Rinse flasks three times with 3 mL of HPLC methanol. Apply to cartridges

8 Add 2-3 mL UV grade acetonitrile to each methanol elegant.

9 Turbo N-evaporate methanol just to dryness.

10 Resuspend residues in 10 mL UV grade ACN:HPLC H_2O (70:30 v/v). Vortex to mix.

11 Microfilterfuge the final extract and aliquot into autosampler vials for analysis on LC/MS, (dilute as necessary).

2.12 LC/MS Analysis of Bensulide

An Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system with electrospray ionization and a Dionex Ultimate 3000 HPLC were used.

2.12.1 LC System Components

SCIEX 4000 (HPLC/Turbo Ion Spray Mode)

Pump: Dionex Ultimate 3000

Autosampler: Dionex Ultimate 3000

Micro-Degasser: Dionex Ultimate 3000

Column Compartment: Dionex Ultimate 3000

2.12.2 LC Parameters

Column: Phenomenex Gemini C6-Phenyl 3.0 µm particle size, 100 Å (50 mm × 2.0 mm ID).

Flow Rate: 300 µL/minute

Injection Volume: 5 µL

Temperature: 40°C

Solvent System: Solvent A = HPLC grade Water (0.1% Formic acid)
Solvent B = HPLC grade Acetonitrile

Solvent Program:

Minutes	Solvent A	Solvent B
0	50	50
0.5	50	50
5.0	5	95
9.0	5	95
9.5	50	50
13.0	50	50

Divert Valve: Divert LC flow from column to waste (bypassing MS) from 0 to 0.1 minutes and again from 12.0 to 13.0 minutes.

Approximate Retention Times: 3.4 minutes,

2.12.3 Mass Spectrometer Parameters

An Applied Biosystems MDS/SCIEX API 4000 tandem mass spectrometer was used with electrospray ionization in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM):

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential	Focusing Potential	Collision Energy	Collision Cell Exit Potential
398.033	158.100	150	36	200	33	10
398.033	218.000	150	36	200	23	16
398.033	141.1000	150	36	200	47	10

Source Dependent Settings:

Nebulizer Temperature (TEM): 450°C

Nebulizer Gas (GS1): 40

Turbo Ionspray Gas (GS2):	7 Liters/min
Ion Spray Voltage (IS):	5500
Curtain Gas (CUR):	10
Interface Heater (ihe):	on
Entrance potential:	10

Separation of the analytes was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of its retention time with that of the respective reference standards as well as by monitoring specific ion transitions.

3.0 METHODS OF CALCULATION

3.1 Preparation of Stock Standards

$$\text{Final Concentration (mg/mL)} = \frac{(W) \times (P)}{(VS)}$$

where W = Milligrams of neat standard
P = Chemical purity of neat standard
VS = Volume of Solvent (mL)

3.2 Residue in Matrices

The analyte was quantified by peak area relative to an external calibration curve. A calibrant peak area (y) from the quantitation ion transition relative to the concentration of the calibrant in ng/mL (x) yielded a linearity curve, where $y = mx + b$ was plotted using a least squares fit with no weighting. Curves are determined by Applied Biosystems/MDS SCIEX Instruments Analyst Software version 1.4.2 or the equivalent.

The residue of Bensulide in water was calculated as follows:

$$\mu g/kg\ (ppb) = \frac{\text{ng/mL analyte} \times \text{Final sample vol. (mL)} \times \times\ 0.001\ \mu g/ng \times \text{Dil factor}}{\text{Sample Wt. (kg)}}$$

Where:

ng/mL analyte	=	ng/mL of analyte found from standard curve
Final sample vol. (mL)	=	Volume of final LC-MS ready sample (10 mL)

Dil Factor	=	1 or 100
0.001 µg/ng	=	Unit conversion factor
Sample weight	=	0.2 kg (200 ml of water)
And:		
ng/mL analyte	=	[(PA – b) ÷ m]
Where:		
PA	=	Peak area analyte
b	=	y-intercept of calibration curve
m	=	slope of calibration curve

$$\% \text{ Recovery} = \frac{\text{Analyte Residue Detected (µg/kg) - Avg. Control Residue}}{\text{Analyte Fortification Level (µg/kg)}} \times 100$$

An example calculation for the bensulide residue in water (Fort 1A at 1 µg/kg) is shown below:

Linear regression analysis (with no weighting) of the Bensulide standards gave a curve as calculated by the Analyst Software version for the quantitation ion transition 398.0/218.0 with the equation

$$y = 21386.513467\ x + 88520.879396\ (r = 0.999541692808)$$

The ng/mL bensulide injected determined by this curve was:

ng/mL bensulide = [((468448.0636 - 88520.879396) ÷ 21386.513467= 17.8 ng/mL

Where:

468448.0636 = peak area bensulide
88520.879396 = y-intercept of calibration curve
21386.513467 = slope

The bensulide residue (mg/kg)

= (17.8 ng/mL x 10.0 mL x 1.0 x 0.001 µg/ng) ÷ (0.2 kg)

= 0.89 µg/kg or ppb

$$\text{Percent Recovery} = \frac{0.89\,\mu g/kg - 0.00\,\mu g/kg}{1\,\mu g/kg} \times 100 = 89\%$$

4.0 LIMIT OF QUANTITATION AND DETECTION

The limit of detection (LOD) is defined as the concentration of the lowest linearity calibrant injected – 4.85 ng/ml Bensulide. Using the current methodology this is equivalent to an LOD of 0.24 ppb. The limit of quantitation (LOQ) is defined as the lowest concentration validated which is 1 ppb for Bensulide.

5.0 STATISTICAL METHODS

The residue data included the following statistical calculations: averages, standard deviations, relative standard deviation and linear regression analysis.

6.0 TIME REQUIRED FOR ANALYSIS

A sample set consisting of 12 samples and a reagent blank sample can be completed by one analyst in the following amount of time:

Extraction/Cleanup: 14 hrs.

Analysis: 13 hr. for analysis (includes 4 hours for instrument tuning).

Total person-hours = 16 (does not include 7 hrs of unattended LC-MS/MS analysis or 4 hrs for tuning)

Days Required for the Sample Set = 2 Days

7.0 ARCHIVING STATEMENT

The original project specific data files will be stored at PTRL-West (Hercules, CA). The project specific data files will be transferred to a location specified by the Sponsor upon their authorization. No data will be discarded without the Sponsor's written consent. Facility records and a copy of the final report are to be maintained at PTRL West.