Cover Sheet for Analytical Method

Benzobicyclon in Water - MRID 49506326

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1.0 INTRODUCTION

Methodology from Morse Laboratories (Meth-221) was validated to quantify the concentration of benzobicyclon and 1315P-070 (metabolite B) present in recovery samples prepared in deionized water, well water and surface water on 22 through 24 May 2013. The detailed Sponsor-supplied method is presented in Appendix 1. This independent laboratory validation (ILV) study is required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one group, can be independently validated by a second group with no major interaction between the two groups. This method was validated by fortification of deionized water, well water and surface water with benzobicyclon and 1315P-070 (metabolite B) at concentrations of 0.001 ppm (LOQ) and 0.010 ppm (10X LOQ). Recovery samples were extracted with 0.05M phosphoric acid and ethyl acetate, concentrated to dryness under nitrogen and reconstituted with 50:50 acetonitrile:purified reagent water. Samples were then analyzed using liquid chromatography with mass spectrometry (LC/MS/MS).

The study was initiated on 17 May 2013, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the ILV study was conducted on 22 through 25 May 2013 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data and the final report produced during this study are archived at Smithers Viscient at the above location.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled “Independent Laboratory Validation (ILV) of the Analytical Method: Determination of Benzobicyclon and 1315P-070 (Metabolite B) in water (Morse Meth-221)”, Smithers Viscient Protocol No.: 120211/ILV/water (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100 for Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA, 2012).

2.2 Test Substances

The test substance, benzobicyclon, was received on 20 August 2012 from PTRL West, Hercules, California. The following information was provided:

Name:	benzobicyclon
Synonym:	3-[2-chloro-4(methylsulfonyl)benzoyl]-4-(phenylthio)bicyclo[3.2.1]oct-3-en-2-one
Lot No.:	1A0110
CAS No.:	156963-66-5
Purity:	>99.9% (Certificate of Analysis, Appendix 2)
Expiration Date:	10 June 2013

Upon receipt at Smithers Viscient, benzobicyclon (SMV No. 5812) was stored frozen in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 1315P-070, was received on 7 March 2013 from PTRL West, Hercules, California. The following information was provided:

Name:	1315P-070
Lot No.:	95Z25
CAS No.:	126656-88-0
Purity:	99.7% (Certificate of Analysis, Appendix 2)
Expiration Date:	14 June 2013

Upon receipt at Smithers Viscient, 1315P-070 (SMV No. 6090) was stored frozen in the original container. Concentrations were adjusted for the purity of the test substance.

Both test substances were used to fortify the recovery samples and prepare the calibration standards during testing. Determination of stability and characterization, verification of the test substances identity, maintenance of records on the test substances, and archival of a sample of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	EMD, reagent grade
2.	Ethyl acetate:	EMD, reagent grade
3.	Sodium sulfate:	EMD, reagent grade
4.	Phosphoric acid:	EMD, reagent grade
5.	Purified reagent water:	prepared from a Millipore Milli-Q® Direct 8 system (meeting ASTM Type II requirements)
6.	Ammonium acetate:	BDH, reagent grade
7.	Methanol:	Burdick & Jackson, reagent grade

2.4 Equipment

1.	Instrument:	AB MDS Sciex 4000 Turbo V ESI with Shimadzu 20AD vacuum degasser, an LC-20AD binary pump, a CBM-20A communication bus, CTO-20AC column oven with a CTO-20AC column compartment, and SIL-20ACHT autosampler; Analyst 1.4.2 for data acquisition
2.	Balance:	Mettler Toledo AG 285
3.	Laboratory equipment:	N-Evap system, volumetric flasks, disposable glass pipets, disposable glass vials, positive displacement pipets, separatory funnels, graduated cylinders, Erlenmeyer flasks, autosampler vials, and amber glass bottles with Teflon®-lined caps

2.5 Preparation of Stock Solutions

A 1000 mg/L primary stock solution was prepared by placing 0.0501 g of benzobicyclon (0.0500 g as active ingredient) in a volumetric flask and bringing it to volume with 50.0 mL of acetonitrile. Two secondary stock solutions (10.0 and 100 mg/L) were prepared by placing 0.500 and 5.00 mL of the 1000 mg/L primary stock solution in separate volumetric flasks and bringing each to volume with 50.0 mL of acetonitrile.

A 1000 mg/L primary stock solution was prepared by placing 0.0502 g of 1315P-070 (0.0500 g as active ingredient) in a volumetric flask and bringing it to a volume of 50.0 mL of acetonitrile.

Two secondary stock solutions (10.0 and 100 mg/L) were prepared by placing 0.500 and 5.00 mL of the 1000 mg/L primary stock solution in separate volumetric flasks and bringing each to volume with 50.0 mL of acetonitrile.

A 0.100 mg/L mixed stock solution was prepared by combining 0.100 mL of the 10.0 mg/L benzobicyclon secondary stock solution with 0.100 mL of the 10.0 mg/L 1315P-070 secondary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 0.100 mg/L mixed stock solution was used to prepare the calibration standards.

A 1.00 mg/L mixed stock solution was prepared by combining 0.100 mL of the 100 mg/L benzobicyclon secondary stock solution with 0.100 mL of the 100 mg/L 1315P-070 secondary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 1.00 mg/L mixed stock solution was used to fortify the low-level recovery samples and prepare the calibration standards.

A 10.0 mg/L mixed stock solution was prepared by combining 0.100 mL of the 1000 mg/L benzobicyclon primary stock solution with 0.100 mL of the 1000 mg/L 1315P-070 primary stock solution in a disposable glass vial and bringing it to a final volume of 10.0 mL with acetonitrile. The 10.0 mg/L mixed stock solution was used to fortify the high-level recovery samples and prepare the calibration standards.

All primary and secondary stock solutions were stored refrigerated in glass amber bottles fitted with Teflon®-lined caps. All sub-stock solutions were prepared daily and discarded after use.

2.6 Reagent Solution and Mobile Phase Preparation

A 50:50 acetonitrile:purified reagent water (v:v) solution was prepared by mixing 100 mL of acetonitrile with 100 mL of purified reagent water. This reagent solution was mixed using a stir bar and stir plate and was used to prepare the calibration standards and dilute the fortified recovery samples.

A 0.05 M phosphoric acid in purified reagent water (v:v) solution was prepared by transferring a portion of purified reagent water to a 500 mL volumetric flask. A 14.8 mL portion of concentrated phosphoric acid was measured and transferred to the volumetric flask containing the purified reagent water. The solution was brought to a final volume of 500 mL with purified reagent water and mixed well. This solution was used to fortify the recovery samples during the ILV.

A 100 mM ammonium acetate in methanol solution was prepared by dissolving approximately 0.77 g of ammonium acetate in 100 mL of methanol. The solution was shaken well to mix. This reagent was used to prepare the mobile phase solutions for LC/MS/MS analysis.

A 19:1 purified reagent water:100 mM ammonium acetate in methanol (v:v) solution was prepared by mixed 950 mL of purified reagent water with 50.0 mL of 100 mM ammonium acetate in methanol. The mobile phase solution was mixed well and degassed under vacuum with sonication.

A 5 mM ammonium acetate in methanol (v:v) solution was prepared by mixing 950 mL of methanol with 50.0 mL of 100 mM ammonium acetate in methanol. The mobile phase solution was mixed well and degassed under vacuum with sonication.

2.7 Preparation of Calibration Standards

Calibration standards were prepared in 50:50 acetonitrile:purified reagent water at concentrations of 0.500 and 1.00 µg/L using the 0.100 mg/L mixed stock solution, at concentrations of 2.50 and 10.0 µg/L using the 1.00 mg/L mixed stock solution and at concentrations of 50.0, 75.0 and 100 µg/L using the 10.0 mg/L mixed stock solution.

2.8 Sample Fortification and Aqueous Preparation

All recovery samples were individually prepared in separatory funnels containing 100 mL of deionized water, well water or surface water at each concentration level by fortification with the appropriate stock solution. Five replicates were prepared at each concentration level in 250 mL separatory funnels in each matrix as follows.

Sample ID	Mixed Stock Concentration (mg/L)	Volume of Stock Solution (mL)	Final Volume (mL)	Fortified Sample Concentration (ppm)
Reagent Blank	NA	NA	NA	0.00
Control A, & B	NA	NA	100	0.00
Low A, B, C, D and E	1.00	0.100	100	0.00100
High A, B, C, D and E	10.0	0.100	100	0.0100

NA = Not Applicable

Two additional 100 mL samples were prepared in each matrix and left unfortified to serve as controls. One additional sample was extracted using only extraction solvents to serve as the reagent blank.

Following fortification, all samples were shaken to mix well. A 10.0 mL aliquot of 0.05 M phosphoric acid solution was then added to each sample followed by 50 mL of ethyl acetate. Each sample was shaken vigorously for approximately five minutes. Following separation of layers, the aqueous and ethyl acetate layers were removed and placed in an Erlenmeyer flask and 500 mL round bottom flasks, respectively. The aqueous layer was then placed back into the separatory funnel and 50 mL of ethyl acetate was used to rinse the flask. The extraction was repeated twice more for a total of three extractions.

Approximately 20 g of sodium sulfate was added to the ethyl acetate extract (approximately 150 mL) and was allowed to sit for 15 minutes, swirling occasionally to mix. The ethyl acetate was then decanted through a glass funnel containing a glass wool plug and approximately 20 g of sodium sulfate into a 250-mL graduated cylinder. The flask was rinsed with 50 mL of ethyl

acetate and added to the graduated cylinder through the glass funnel. The extract was then brought to a final volume of 200 mL with ethyl acetate, with the exception of a deionized water low-level recovery sample that was inadvertently taken to a volume of 210 mL. The extracts were transferred to a 250-mL HDPE centrifuge bottle and mixed well. A 10 mL aliquot of the extract was then transferred to a graduated glass conical and concentrated to approximately 0.2 mL using an N-Evap under a gentle stream of nitrogen in a water bath at 40 °C, followed by further evaporation to dryness under a gentle stream of nitrogen at room temperature. The sample was then reconstituted to a final volume of 1.0 mL with 50:50 acetonitrile:purified reagent water, vortex mixed for 30 seconds and sonicated for 5 minutes. Samples were transferred to amber GC vials with crimp caps and analyzed by LC/MS/MS. A typical dilution is described below.

Sample ID	Nominal Concentration (ppm)	Sample Volume (mL)	Final Volume[a] (mL)	Sub-sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Reagent Blank	0.00	NA	200	10.0	1.00	0.200
Control A, & B	0.00	100	200	10.0	1.00	0.200
Low[c] A, B, C, D and E	0.00100	100	200	10.0	1.00	0.200
High A, B, C, D and E	0.0100	100	200	10.0	1.00	0.200

[a] Diluted to volume with ethyl acetate.
[b] Diluted to volume with 50:50 acetonitrile: purified reagent water
[c] The low-level replicate A for the deionized water matrix was inadvertently brought to a final volume of 210 mL with ethyl acetate, therefore the dilution factor is 0.0210.

2.9 Analysis

2.9.1 Instrumental Conditions

The LC/MS/MS analysis was conducted utilizing the following instrumental conditions;

Column:	Phenomenex Luna C18, 3µm, 150 x 2.0 mm
Temperature:	40 °C
Mobile Phase A:	19:1 purified reagent water:100 mM ammonium acetate in methanol, gradient analysis
Mobile Phase B:	5 mM ammonium acetate in methanol, gradient analysis

Flow Rate: 0.20 mL/minute
Run Time: 13 minutes
Injection Volume: 2.0 µL
Retention Time: Approximately 7.9 minutes (benzobicyclon)
Approximately 5.9 minutes (1315P-070)

Type	Condition	Parameter
MS	Source temperature	500 °C
	Scan type	MRM
	Ionization Mode	Positive
	Ion Source	Turbo Spray
	Ion spray voltage	5000.00
	Dwell time	150.00 msec
	Curtain Gas	10.00
	Ion source- gas 1/gas 2	20.00/20.00
	Collision Gas	4.00
	Collision Cell Entrance Potential	10.00
	Interface Heater	On

Transitions Monitored:

Compound	Ion[a] (amu)	Collision Energy	Collision Exit Potential	Declustering Potential
Benzobicyclon	447.08/257.20	35.00	16.00	76.00
Benzobicyclon	447.08/229.20	51.00	16.00	76.00
Benzobicyclon	447.08/139.10	107.00	8.00	76.00
1315P-070	355.14/165.20	33.00	10.00	101.00
1315P-070	355.18/183.10	31.00	12.00	101.00
1315P-070	355.18/319.00	31.00	12.00	101.00
1315P-070	355.18/69.20	77.00	12.00	101.00
1315P-070	355.18/81.10	63.00	14.00	101.00

[a] Ions are presented as Q1 mass/Q3 mass

Gradient Tables:

Time (min)	A (%)	B (%)
1.0	80.0	20.0
3.0	20.0	80.0
9.0	20.0	80.0
10.0	80.0	20.0
13.0	80.0	20.0

2.9.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery

samples. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.9.3 Method Differences

There was no method differences between Meth-221 and the procedure performed at Smithers Viscient.

2.10 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the low- and high-level recovery samples. Recoveries of 70 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the low- and high-level recovery samples for each analyte. The retention time should have an RSD of less than or equal to 2%. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as benzobicyclon and 1315P-070 which might interfere with the quantitation of analytes. Interferences with peak areas that are less than 50% at the limit of detection (LOD) are not considered significant. Linearity of the method was determined by the correlation coefficient (r^2), y-intercept and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient not less than 0.990. The precision of the method at the LOQ was reported in terms of the relative standard deviation or coefficient of variation of the observed recovery values.

2.11 Communications

Communications occurred with the Sponsor Monitor to discuss items such as
1) clarification/approval of the protocol and method, 2) acquisition of analytical standard and

control sample, and 3) pre-validation evaluation and method establishment including calibration curve linearity. A complete list of communications is maintained in the study raw data.

2.12 Time Required for Analysis

A normal batch of samples consists of 10 fortified and 2 unfortified samples, 1 matrix-match blank and 7 solvent standards (20 samples total) for each matrix. A single analyst completed a set of 20 samples in one working day (8 hours) with LC/MS/MS analysis performed overnight.

3.0 Calculations

A calibration curve was constructed by plotting the analyte concentration (µg/L) in the calibration standards against the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the peak area of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = ax^2 + bx + c$$

$$(2) \qquad DC(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

$$(3) \qquad A = DC \times DF$$

where:

y	=	detector response (peak area) for analyte
a, b and c	=	regression constants
DC (x)	=	detected concentration (µg/L) in the sample
C	=	constant c minus the peak area; C = (c - y)
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The method limit of detection (LOD) was calculated using the following equation (U.S. EPA, 2000):

$$(4)\ LOD = t_{0.99} \times s$$

where:

$t_{0.99}$ = t value for n-1 replicates at the 99% confidence interval
s = standard deviation (µg/L) for the method LOQ (where the LOQ = 1.00 µg/L)