1.0 ABSTRACT

Method Validation for Niclosamide in Ecotoxicology Media

This report describes the validation of an analytical method for the analysis of niclosamide in various media that will be used during ecotoxicological testing. The method validation utilized a high-performance liquid chromatography system equipped with a UV detector and a Waters Symmetry C18 column. Quantitation was performed by external standard calibration using peak areas.

The method was shown to be valid for the analysis of niclosamide in the three ecotoxicology media (freshwater, 20X FWAM, and sediment) tested. The accuracy, precision, recovery, and linearity data have shown this method to be acceptable for niclosamide. Sample solution stability after at least seven days refrigerator storage was acceptable for niclosamide.

2.0 INTRODUCTION

The objective of this study was to validate the method for the analysis of niclosamide in various media that will be used during ecotoxicological testing. The study was conducted as described in the ABC study protocol titled "Method Validation for niclosamide in Ecotoxicology Media," which was patterned after the European Commission Working Document SANCO/3030/99 rev. 4 (1).

This report accurately reflects what was actually performed during the course of the study.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Test And Reference Substance

ABC Laboratories received the test substance niclosamide (Lot No.: BCBG9789V) from Sigma-Aldrich on 05 June 2014. The test substance was given the ABC designation of MM-10331-00001 and was stored at room temperature. A non-GLP Certificate of Analysis was provided with the test substance. ABC Laboratories' subsequently determined the purity of the test substance to be 100% under GLP conditions (Appendix A), and a recertification date of 25 September 2016 was assigned.

3.1.2 Test Systems

3.1.2.1 Freshwater Test Media

Freshwater was prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis (RO). The well water and RO water were blended together to yield a total hardness of 130 to 160 mg/L as $CaCO_3$. Characterization of the base water, i.e., ABC well water, used to prepare the dilution water can be found in Appendix B.

3.1.2.2 20X Freshwater Algal Nutrient Medium (20XAAP)

The 20XAAP medium (2) was prepared by the addition of appropriate reagent grade salts to autoclaved ABC reagent water. ABC reagent water is produced by passing reverse-osmosis water through a series of deionization tanks, a laboratory water purification system consisting of carbon, de-mineralization, and organic adsorption cartridges, and then through a 0.2-µm filter. After preparation, the medium was adjusted to pH 7.5 ± 0.1 with 0.1N HCl and filtered through Millipore 0.45-µm filters. Chemical characterization of a representative sample of ABC well water, the base water used to prepare ABC reagent water, is presented in Appendix B.

3.1.2.3 Sediment

The formulated sediment (Lot No.: ASD190614) was prepared by mixing 7500 g of sand, 2000 g of clay, and 588.4 g of peat together, resulting in a component composition (% dry weight) of 75, 20, and 5 for sand, clay, and peat, respectively. The pH of the formulated sediment was 6.82. To

produce 35% moist artificial sediment, 250.232 g artificial sediment was mixed with 83.638 g deionized water.

3.1.3 Reagents

All reagents employed in this study were ACS reagent grade or purer.

3.1.4 Equipment

- Balance: Mettler XP205DR; Mettler BP3260; Mettler PM400; Mettler MS 1003S
- pH meter: WTW Model pH 330i
- Water quality meter: WTW Multi 3500i
- Moisture analyzer: Mettler HR73 Halogen Moisture Analyzer
- Centrifuge: IEC Centra-HN
- Sciex 4000 liquid chromatographic/mass spectrometry (LC-MS/MS) system
- Agilent LC-UV system
- LC column: Waters Symmetry C18
- Volumetric glassware
- Miscellaneous laboratory glassware
- Refrigerators/Freezers: Mr. Winter Refrigerator; True Refrigerator GDM-40; Labline Refrigerator/Freezer

3.2 Methods

The study was conducted as described in the ABC protocol entitled, "Method Validation for Niclosamide in Ecotoxicology Media" and amendment (Appendix C).

3.2.1 Preparation of Analytical Standard and Matrix Spiking Solutions

A primary stock solution of niclosamide was prepared on 25 July 2014 by dissolving 10.9 mg niclosamide in 100 mL of methanol, resulting in a concentration of 0.109 mg/mL. Subsequent dilutions of this primary stock solution in 20:80 methanol:water were used as calibration standards.

Another primary stock solution of niclosamide was prepared on 25 July 2014 by dissolving 31.4 mg niclosamide in 50 mL of methanol, resulting in a concentration of 0.628 mg/mL. This primary stock solution and a subsequent dilution in methanol were used as fortification solutions.

A third primary stock solution of niclosamide was prepared on 29 July 2014 by dissolving 101.6 mg niclosamide in 50 mL methanol, resulting in a concentration of 2.03 mg/mL. This primary stock solution was used as a fortification solution.

All solutions were stored refrigerated when not in use.

3.2.2 Sample Analysis

Water sample analysis was accomplished by dilution of samples with methanol and further dilution, if necessary, with 20:80 methanol:water, followed by direct analysis performed on an LC-UV system. Sediment sample analysis was accomplished by extraction of samples with methanol followed by dilution of the concentrated extracts. Analysis was performed on an LC-UV system.

3.2.3 Instrument Conditions

Instrument:	Agilent 1100 LC with variable wavelength detector
Isocratic Mobile Phase:	15:85 58mM acetate buffer in water:methanol
Flow Rate:	1.0 mL/min
Injection Volume:	50 µL
Column:	Waters Symmetry C18, 3.5 µm, 75 mm x 4.6 mm
Column Temp:	25 °C
Wavelength:	335 nm

Note: Instrument conditions may be changed to optimize chromatography.

3.2.4 Calculations

Calculation of niclosamide concentrations in test solution samples analyzed by LC-UV were performed by the external standard analysis function of Empower 2 software. The concentration of the analyte in each sample was determined directly from the standard curve by the following equation:

$$\frac{(\text{Concentration from standard curve in mg/L})(\text{analysis volume in mL})}{\text{sample volume (or mass) in mL (or g)}} = \text{mg/L (or mg/kg)}$$

The standard curve equation is of the form: $y = mx + b$

where:

- y = peak area units
- m = slope of the standard curve [X Coefficients(s)]
- x = mg of niclosamide/L
- b = y-intercept (Constant)

Example calculation for the Low Spike LOQ 1 sample in the 20XAAP method validation:

Standard Curve: y = 176,000x - 189
Sample Peak Area: 2,906
Concentration from standard curve: 0.0176 ng/mL

Volume for Analysis: 12.5 mL
Sample Volume: 10 mL

The concentration of niclosamide in the sample was calculated by the following equation:

$$\frac{(0.0176\ \text{mg/L})(12.5\ \text{mL})}{10\ \text{mL}} = 0.0220\ \text{mg/L}$$

Recovery from the Low Spike LOQ 1 sample in the 20XAAP method validation:

$$\frac{0.0220\ \text{mg/L}}{0.0209\ \text{mg/L}} \times 100 = 105\%$$

The minimum quantifiable limit (MQL) was determined from the following equation:

$$\frac{\left(\begin{array}{c}\text{lowest standard}\\ \text{concentration as mg/L)}\end{array}\right)\left(\begin{array}{c}\text{volume for}\\ \text{analysis (mL)}\end{array}\right)}{\left(\begin{array}{c}\text{volume or mass}\\ \text{sampled (mL or g)}\end{array}\right)} = \text{MQL expressed as mg/L or mg/kg}$$

Example for 20XAAP method validation:
Lowest standard concentration: 0.00500 mg/L
Analysis volume: 10 mL
Sample volume: 8 mL

therefore:

$$\text{MQL} = \frac{(0.00500\ \text{mg/L})(10\ \text{mL})}{(8\ \text{mL})} = 0.00625\ \text{mg/L}$$

3.2.5 Linearity

A 6-point calibration was prepared for each analysis and slope, intercept, and correlation coefficient were determined. The correlation coefficient was used to assess the linearity of the standard curves. Section 3.2.1 describes the preparation of the standard solutions.

3.2.6 Method Validations in Test Media

3.2.6.1 Freshwater

Method validations for the recovery of niclosamide in freshwater were performed on 27 July and 28 August 2014. On 27 July 2014, five samples of 8 mL volume freshwater were fortified with 0.200 mL of a 0.836 mg/L solution for a nominal concentration of 0.0209 mg/L (low spikes), and five samples of 8 mL freshwater were fortified with 0.800 mL of a 628 mg/L solution for a nominal concentration of 62.8 mg/L (high spikes). Additionally, one sample of 8 mL freshwater was fortified with 0.060 mL of a 0.836 mg/L solution for a nominal concentration of 0.00627 mg/L (LOD spike). The remaining two samples consisted of matrix only (i.e., control freshwater). The samples were diluted with methanol and further diluted, if necessary, with

20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

On 28 August 2014, five samples of 8 mL freshwater were fortified with 0.500 mL of a 2,030 mg/L solution for a nominal concentration of 127 mg/L (additional high spikes). The samples were diluted with methanol and further diluted with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

3.2.6.2 20XAAP

Method validations for the recovery of niclosamide in 20XAAP were performed on 28 and 29 July and 28 August 2014. On 28 July 2014, five samples of 8 mL 20XAAP were fortified with 0.250 mL of a 0.836 mg/L solution for a nominal concentration of 0.0209 mg/L (low spikes). Additionally, one sample of 8 mL 20XAAP was fortified with 0.075 mL of a 0.836 mg/L solution for a nominal concentration of 0.00627 mg/L (LOD spike). The remaining two samples consisted of matrix only (i.e., control 20XAAP). The samples were diluted with methanol to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

On 29 July 2014, five samples of 8 mL 20XAAP were fortified with 0.250 mL of a 2,030 mg/L solution for a nominal concentration of 63.4 mg/L (high spikes). The samples were diluted with methanol and further diluted with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

On 28 August 2014, five samples of 8mL 20XAAP were fortified with 0.500 mL of a 2,030 mg/L solution for a nominal concentration of 127 mg/L (additional high spikes). The samples were diluted with methanol and further diluted with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

3.2.6.3 Sediment

The method validation for the recovery of niclosamide in sediment was performed on 06 August 2014. Thirteen samples of approximately 1 g dry sediment (approximately 1.37 g wet sediment) were used. Five samples were fortified with 0.239 mL of a 0.836 mg/L solution for a nominal concentration of 0.200 mg/kg (low spikes), and five samples were fortified with 0.100 mL of a 2,030 mg/L solution for a nominal concentration of 203 mg/kg (high spikes). Additionally, one sample (LOD spike) was fortified with 0.072 mL of a 0.836 mg/L solution for a nominal concentration of 0.0602 mg/kg. The remaining two samples consisted of matrix only (i.e., control sediment).

Samples were vortexed with 2 mL methanol, then shaken for 30 minutes. Following shaking, the samples were centrifuged for 10 minutes at 3400 rpm. The supernatant was transferred to a separate culture tube, then the extraction with methanol and centrifugation repeated twice more. Following the extractions, the methanol extracts were evaporated under nitrogen to 2 mL. Eight

(8) mL HPLC water were added to each sample. If necessary, samples were further diluted with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to HPLC vials for analysis by LC-UV.

3.2.7 Storage Stability

Three replicates of the low spike level (0.0209 mg/L) and three replicates of the high spike level (62.8 mg/L) from the freshwater method validation were analyzed after eleven days of refrigerator storage. Three replicates of the low spike level (0.0209 mg/L) and three replicates of the high spike level (63.4 mg/L) from the 20XAAP method validation were analyzed after at least nine days of refrigerator storage. Three replicates of each low spike level (0.200 mg/kg) and high spike level (203 mg/kg) from the method validation samples in sediment were analyzed after seven days of refrigerator storage. Stability of test substance parent and intermediate stock solutions was verified by the repeated acceptable recovery of spikes during the other phases of the study.

3.2.8 Statistics

Calculations (e.g., percent area, percent difference, mean, and standard deviation) were performed using Microsoft Excel 2007, and intermediate values were not rounded during the calculations. Since Microsoft Excel was run in full precision mode, values represented in the raw data and report may be slightly different when calculated by hand.

4.0 RESULTS AND DISCUSSION

4.2 MDL and PQL

The MDL and PQL were determined using seven replicate samples of the lowest calibration standard injected.

The MDL and PQL for niclosamide were determined to be 0.000606 and 0.00303 mg/L, respectively (Table 1).

4.4 Storage Stability

The intent of determining storage stability was to assess if samples could be stored for at least seven days prior to analysis without biasing the results of their analysis. Instability in this case is considered to be loss from initial measurements greater than 20%. Regardless of specific storage stability results, analysis of samples from ecotoxicity studies is recommended to be done as soon as possible after sampling (i.e., without storage if possible).

Table 1. Method Detection Limit (MDL) and Practical Quantitation Limit (PQL) of Niclosamide

Sample Identification	Nominal Concentration (mg/L)	Measured Concentration (mg/L)
CDG 13281-A7	0.00500	0.004961
CDG 13281-A7	0.00500	0.005075
CDG 13281-A7	0.00500	0.005149
CDG 13281-A7	0.00500	0.005257
CDG 13281-A7	0.00500	0.005249
CDG 13281-A7	0.00500	0.005440
CDG 13281-A7	0.00500	0.005504
	MEAN	0.00523
	STDEV	0.000193
	MDL = 3.14XSTDEV	0.000606
	PQL = 5XMDL	0.00303