STUDY SUMMARY

The objective of this study was to validate the method for the analysis of ipconazole in saltwater media that will be used during ecotoxicological testing. Water samples were extracted using C_{18} solid phase extraction (SPE) cartridges. The cartridges were eluted with acetonitrile which was then reduced to near dryness under a steady stream of nitrogen and reconstituted in methanol. Further dilutions were prepared using HPLC water, if required. Samples were quantified using a liquid chromatography system with tandem mass spectrometry (LC-MS/MS).

The accuracy, precision, recovery, and linearity data have shown this method to be acceptable for ipconazole, based on the analysis of ipconazole cc and ipconazole ct active ingredients, in the saltwater ecotoxicology media tested. The limit of quantitation (LOQ) of the method was 0.0500 µg/L. Sample solution stability after at least eight days refrigerated storage in saltwater was acceptable for ipconazole. The stability of ipconazole in calibration standards was up to 25 days in refrigerated storage.

This report satisfies the data requirement for EU SANCO\3029\99 rev.4.

REASON FOR REPORT REVISION

Report Revision No. 1

This report has been revised as follows:

– A compliance exception concerning the recording of the use of an evaporator and vacuum was added to the Compliance with Good Laboratory Practice Standards page.

– Reagents were fully detailed in Section 2.1.2 Reagents

– SPE cartridge and pipette identification were added to Section 2.1.4 Equipment.

– Steps for sample preparation were added to Section 2.2.2 Sample Analysis.

– More details of instrument conditions were added to Section 2.2.3 Instrument Conditions.

1.0 INTRODUCTION

The objective of this study was to validate the method for the analysis of ipconazole in saltwater media that will be used during ecotoxicological testing. This test protocol is intended to support environmental toxicity studies conducted in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals. The method validation will be conducted in accordance with the SANCO/3029/99 rev 4 guidelines (1) and U.S. EPA ecological effects testing guideline OCSPP 850.6100 (2).

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

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Test and Reference Substances

A sample of the test substance, Ipconazole TG, (Batch No. 89010; CAS number 125225-28-7) was received from Kureha Corporation on 17 May 2017 and was stored ambient. The sample was assigned reference number MM-19189-00001. The expiration date was 24 November 2019. The test substance was characterized under Good Laboratory Practices, and the Certificate of Analysis listed the purity as 96.7% w/w, containing 89.7% w/w ipconazole cc and 7.0% w/w ipconazole ct (Appendix A). This material was used to prepare matrix spiking solutions.

A sample of the reference substance, Ipconazole cc, (Lot No. G-00328) was received from Kureha Corporation on 17 May 2017 and was stored ambient. The sample was assigned reference number MM-19190-00001. The expiration date was 12 September 2021. The Certificate of Analysis described the reference substance as a white powder with a purity 99.5% (Appendix A). This material was used to prepare analytical standard solutions.

A sample of the reference substance, Ipconazole ct, (Lot No. G-00329) was received from Kureha Corporation on 17 May 2017 and was stored ambient. The sample was assigned reference number MM-19191-00001. The expiration date was 09 September 2021. The Certificate of Analysis described the reference substance as a white powder with a purity 99.7% (Appendix A). This material was used to prepare analytical standard solutions.

2.1.2 Reagents

Reagents used in the study are as follows:

Acetonitrile – Fisher Chemical, Lot Nos. 165489, 170636, 173555

Methanol – Fisher, Lot Nos. 162838, 165089, 166751, 167576, 170575, 171470

HPLC Grade Water – Fisher, Lot Nos. 167209, 168193, 170390, 172274, 172276

Isopropyl Alcohol – Fisher, Lot No. 151391

Formic Acid – Fisher, Lot No. 154970

Ammonium Formate – Fisher, Lot No. 157840

Octan-1-ol – Acros Organics, Lot No. A0374508

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

2.1.3 Saltwater Test Media

The laboratory saltwater was prepared by adding a commercial sea salt mix (Crystal Sea Marinemix; Marine Enterprises International, Inc. Baltimore, Maryland) to laboratory freshwater at a target salinity 20 ± 3‰. The laboratory freshwater consists of on site well water blended with well water that was demineralized by reverse osmosis to yield water with a total hardness ranging from 130 to 160 mg $CaCO_3$/L. The measured pH of the laboratory saltwater was 8.10. The most recent screen of the laboratory saltwater for selected chemical parameters and potential contaminants is presented in Appendix B.

2.1.4 Equipment

Balances:	Mettler XP205DR
Solid phase extraction (SPE) cartridges	Waters Sep-pak C_{18} 6cc Vac Cartridge, 1 g Sorbent per Cartridge
Pipettes:	Rainin 3-25µL, Rainin 10-100µL, Rainin 20-50µL, Rainin 50-250µL, Rainin 100-1000µL
Evaporator:	Zymark Turbovap LV
Glassware:	Class A volumetric unless otherwise noted
LC-MS/MS:	Waters Acquity UPLC System equipped with AB Sciex API 5500 Q-Trap tandem mass spectrometer (MS) detection

2.2 Methods

The study was conducted as described in the protocol entitled, "Method Validation for Analysis of Ipconazole in Salt Water," with amendments and deviation (Appendix C).

2.2.1 Preparation of Matrix Spiking Solutions and Analytical Standards

A primary stock solution of the test substance, ipconazole TG, was prepared on 28 June 2017 by weighing 93.3 mg of ipconazole into a volumetric flask and diluting to a 25-mL volume with acetonitrile (ACN) for a concentration of 3.61 mg/mL, corrected for purity. This primary stock solution and subsequent dilutions in ACN were used to prepare spiking (fortification) solutions.

A primary stock solution of the reference substance, ipconazole cc, was prepared on 28 June 2017 by weighing 10.3 mg of ipconazole cc into a volumetric flask and diluting to a 25-mL volume with ACN for a concentration of 0.410 mg a.i./mL, corrected for purity. Subsequent dilutions of this primary stock solution in 50:50 HPLC water:methanol were used to prepare analytical standard solutions.

A primary stock solution of the reference substance, ipconazole ct, was prepared on 28 June 2017 by weighing 10.6 mg of ipconazole ct into a volumetric flask and diluting to a 25-mL volume with ACN for a concentration of 0.423 mg a.i./mL, corrected for purity. Subsequent dilutions of this primary stock solution in 50:50 HPLC water:methanol were used to prepare analytical standard solutions.

All solutions were stored refrigerated when not in use.

2.2.2 Sample Analysis

For each analysis, a 20-mL sample was collected and placed into a 20-mL culture tube. The samples were fortified with spiking solutions as necessary, removing an aliquot equal in volume to the fortification volume. One milliliter of ACN was added to each sample and C_{18} SPE cartridges were conditioned by passing 5 mL of ACN followed by 5 mL of HPLC water through to waste, not allowing the packing to become dry. Samples were then loaded on to the cartridges at a steady rate, by gravity, discarding the eluate. A 5-mL volume of HPLC water was then added to rinse the culture tube, and cartridges washed with the water, discarding the eluate. Air was then drawn through the cartridges for a few minutes, by vacuum, to remove any residual water. The cartridges were eluted by gravity with 8 mL of ACN, collecting the eluate in a 10-mL culture tube and air was then drawn through the cartridges for a few minutes, by vacuum, to remove any residual ACN. A 20 µL volume of octan-1-ol was added to the eluate and then blown down to near dryness under a steady stream of nitrogen at approximately 45 °C in an evaporator. The samples were then reconstituted in 1 mL of methanol with the aid of vortex mixing and ultra-sonication. Finally, samples were diluted to a volume of 2 mL with HPLC water, vortexed, and stored refrigerated until analyzed using the instrument conditions listed below.

Sample analysis was performed using a liquid chromatography system with tandem mass spectrometry (LC-MS/MS) for saltwater samples. Data was acquired using Analyst 1.6.2, and the resulting values transcribed to an Excel spreadsheet for calculations.

2.2.3 Instrument Conditions

Sample analysis was performed using a LC-MS/MS system equipped with the following analytical parameters:

Instrument:	AB Sciex API 5500 Q-Trap
Column:	Phenomenex Biphenyl, 100 mm × 4.6 mm, 2.6 µm
Ionization:	TurboIon Spray
Mobile Phase A:	0.1 mM Formic acid + 0.1 mM Ammonium formate (aq)
Mobile Phase B:	Methanol

Gradient:

Time (min)	%A	%B
0.00	30	70
1.00	30	70
8.00	25	75
8.01	5	95
10.0	5	95
10.1	30	70
12.5	30	70

Flow Rate:	0.800 mL/minute
Column Temperature:	40°C
Injection Volume:	2.00 or 5.00 µL (ipconazole cc), 10.0 or 20.0 µL (ipconazole ct)
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	20.00
Collision Gas (CAD):	Medium
Temperature (TEM):	500 °C
Ion Source Gas 1 (GS1):	40.00
Ion Source Gas 2 (GS2):	50.00
IonSpray Voltage (IS):	5500.00 volts
Entrance Potential (EP):	10.00 volts
Collision Energy (CE):	60.00 volts
Declustering Potential (DP):	60.00 volts
Cell Exit Potential (CXP):	8.00 volts

	Ions		
Compound	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)
Ipconazole cc	334.100	70.100	80.00
Ipconazole cc a (confirmatory ion)	334.100	125.000	80.00
Ipconazole ct	334.101	70.100	80.00
Ipconazole ct a (confirmatory ion)	334.101	125.000	80.00

Note: Instrument conditions may be changed to optimize chromatography.

2.2.4 Calculations

Calculations of ipconazole concentrations, based on ipconazole cc and ipconazole ct analysis, in test solution samples were performed by the external standard analysis function of Analyst 1.6.2 software. The concentration of ipconazole cc and ipconazole ct from each sample was determined directly from the standard curve by the following equation:

$$\frac{(\text{ng a.i./mL from standard curve})(\text{analysis volume in mL})}{\text{sample volume in mL}} = \text{ng a.i./mL}$$

Ipconazole cc

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 = ng a.i./mL
b = y-intercept (Constant)

Example calculation for the 0.0500 µg/L low spike B method validation sample:

Standard Curve: $y = 767{,}096.2x + 4{,}387.425$
Sample Peak Area: 348,909
Concentration from standard curve: 0.44912 ng a.i./mL

Volume for Analysis: 2 mL
Sample Volume: 20 mL

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

$$\frac{(0.44912 \text{ ng a.i./mL})(2 \text{ mL})}{20 \text{ mL}} = 0.044912 \text{ ng a.i./mL} = 0.044912 \text{ µg a.i./L}$$

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

$$\frac{\left(\begin{matrix}\text{lowest standard} \\ \text{concentration (µg/L)}\end{matrix}\right)\left(\begin{matrix}\text{volume for} \\ \text{analysis (mL)}\end{matrix}\right)}{(\text{volume sampled (mL)})} = \text{MQL expressed as µg/L}$$

Lowest standard concentration: 0.100 µg/L
Volume for analysis: 2 mL
Volume of sample: 20 mL

therefore:

$$\text{MQL} = \frac{(0.100\ \mu g/L)(2\ mL)}{20\ mL} = 0.0100\ \mu g/L$$

Ipconazole ct

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 = ng a.i./mL
b = y-intercept (Constant)

Example calculation for the 0.0500 µg/L low spike B method validation sample:

Standard Curve: $y = 4{,}420{,}196x - 1{,}361.312$
Sample Peak Area: 160,484
Concentration from standard curve: 0.03662 ng a.i./mL

Volume for Analysis: 2 mL
Sample Volume: 20 mL

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

$$\frac{(0.03662\ \text{ng a.i./mL})(2\ mL)}{20\ mL} = 0.003662\ \text{ng a.i./mL} = 0.003662\ \mu g\ \text{a.i./L}$$

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

$$\frac{\left(\begin{matrix}\text{lowest standard} \\ \text{concentration } (\mu g/L)\end{matrix}\right)\left(\begin{matrix}\text{volume for} \\ \text{analysis (mL)}\end{matrix}\right)}{(\text{volume sampled (mL)})} = \text{MQL expressed as } \mu g/L$$

Lowest standard concentration: 0.0100 µg/L
Volume for analysis: 2 mL
Volume of sample: 20 mL

therefore:

$$\text{MQL} = \frac{(0.0100\ \mu g/L)(2\ mL)}{20\ mL} = 0.00100\ \mu g/L$$

The results of the ipconazole cc and ipconazole ct analyses were added together to determine the concentration of ipconazole. Continuing with the 0.0500 µg/L low spike B method validation sample example:

0.044912 µg ipconazole cc a.i./L + 0.003662 µg ipconazole ct a.i./L = 0.0486 µg ipconazole/L

Recovery of 0.0500 µg/L low spike B method validation sample:

$$\frac{0.0486\ \mu g/L}{0.0500\ \mu g/L} \times 100 = 97\%$$

2.2.5 Calibration Curve

A 7-point calibration was prepared for each ipconazole cc and ipconazole ct analysis and slope, intercept, and correlation coefficient were determined. The correlation coefficient was used to assess the standard curves. Section 2.2.1 details the preparation of the standard solutions.

2.2.6 Method Validations in Saltwater

Method validation for the recovery of ipconazole, based on ipconazole cc and ipconazole ct analysis, in saltwater was performed from 13 to 14 July 2017. Thirteen 20-mL volumes of saltwater were collected in 20-mL glass culture tubes. Five samples (low spikes) were fortified with 0.0500 mL of a 0.0200 mg/L ipconazole solution for a nominal concentration of 0.0500 µg/L. This low spike concentration was considered the limit of quantitation (LOQ). Five samples (high spikes) were fortified with 0.100 mL of a 0.100 mg/L ipconazole solution for a nominal concentration of 0.500 µg/L. The remaining three samples consisted of saltwater only (i.e., control water). A reagent blank was also prepared using HPLC water.

2.2.7 Storage Stability

Saltwater fortified with ipconazole at concentrations of 0.0500 and 0.500 µg/L from the method validation test were analyzed for the ipconazole cc active ingredient after eight days of refrigerated storage (5 ± 3°C) and for the ipconazole ct active ingredient after 12 days of refrigerated storage (5 ± 3°C).

2.2.8 Stability in Calibration Standards

Stability of the ipconazole cc and ipconazole ct active ingredients was performed on 07 August 2017. Two ipconazole cc standard solutions (0.100 and 1.00 µg a.i./L), prepared per Section 2.2.1 above, were removed from refrigerated storage (5 ± 3°C) after 25 days of storage and analyzed for ipconazole cc. Two ipconazole ct standard solutions (0.0200 and 0.200 µg a.i./L), prepared per Section 2.2.1 above, were removed from refrigerated storage (5 ± 3°C) after 25 days of storage and analyzed for ipconazole cc.