ABSTRACT

A study was conducted to provide an independent laboratory validation of a method for the determination of residues of pyroxasulfone[5] (KIH-485), and its metabolites KIH-485 M-1 and KIH-485 M-3 (herein referred to as KIH-485, M-1 and M-3), in freshwater. The validation sample set consisted of two controls and ten fortified samples of freshwater where five replicate subsamples were fortified at the limit of quantitation (LOQ) and five at ten fold the LOQ with a mixed standard of KIH-485, M-1 and M-3. The LOQ was 0.005 mg/L for KIH-485, M-1 and M-3 in water. Samples were analyzed by the method presented with final quantitation of analytes performed by liquid chromatography with mass spectral detection (LC/MS/MS).

INTRODUCTION

Wildlife International, Ltd. performed an independent laboratory validation (ILV) of an analytical method for the determination of pyroxasulfone[5] (KIH-485), and its metabolites M-1 and M-3, in freshwater. The protocol for this study titled "Independent Laboratory Validation of a Method for the Analysis of Pyroxasulfone[5], and its Metabolites M-1 and M-3 in Water" is presented in Appendix I. The analytical method, KIH-485, M-1 and M-3 Water Method as Described in "Aquatic Field Dissipation of Residues Following Application of KIH-485 WG85 to Water" is presented in Appendix II.

This study was performed to satisfy regulatory requirements for independent laboratory validation of methods as set forth by the U.S. Environmental Protection Agency Series 860 - Residue Chemistry Test Guidelines, OPPTS 860.1340, *Residue Analytical Method* (1) and U.S. Environmental Protection Agency, 1996. Pesticide Regulation (PR) Notice 96-1: Notice to Manufacturers, Formulators, Producers and Registrants of Pesticides Products, *Tolerance Enforcement Methods - Independent Laboratory Validation By Petitioner*. (2). The study was performed at the Wildlife International, Ltd. analytical chemistry facility in Easton, Maryland. The experimental portion of the study was conducted between October 17 and October 21, 2008. Raw data and a copy of the final report are archived at the Wildlife International, Ltd. site.

PURPOSE

This study was conducted to fulfill EPA requirements set forth in guideline OPPTS 860.1340 and PR Notice 96-1. This study provides validation data demonstrating that an independent researcher could reproduce the results of the analytical method with minimal contact with the method developers.

[5] ISO 1750 approved name for KIH-485.

EXPERIMENTAL DESIGN

Freshwater was fortified with KIH-485, and its metabolites M-1 and M-3, at two different concentrations and analyzed according to the method supplied by the Sponsor. The lower concentration was at the method LOQ. Matrix blanks (controls) were analyzed concurrently to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Freshwater - Origin

Freshwater was obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water was passed through a sand filter and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water was filtered to 0.45 µm in order to remove fine particles. The water is characterized as moderately hard. Average values for the approximate four-week period, prior to use in this method validation study, for hardness, alkalinity, pH and specific conductance were as follows:

Hardness as calcium carbonate ($CaCO_3$)	137 mg/L
Alkalinity as $CaCO_3$	179 mg/L
pH	8.1
Specific Conductance	362 µmhos/cm

Test and Reference Substances

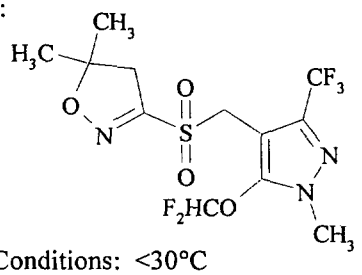
Pyroxasulfone, identified as KIH-485 (PAI) in transmittal correspondence, M-1 and M-3 were received from LSG Corporation. Pyroxasulfone was received on March 24, 2006 and assigned Wildlife International, Ltd. Identification Number 7561. Its metabolites M-1 and M-3 were received on March 11, 2008 and assigned Identification Numbers 8423 and 8424, respectively.

These substances served as both test substances for sample fortification and reference substances for subsequent quantitation. Pyroxasulfone was transferred to ambient storage in darkness upon receipt whereas M-1 and M-3 were transferred to refrigerated storage in darkness. A Certificate of Analysis and Statement accompanied each test substance and provided the following physicochemical and characterization information (Appendix III). The structure of pyroxasulfone was obtained from additional information supplied via Kumiai Chemical Industry Co., Ltd. (3).

Pyroxasulfone (KIH-485)

Chemical Name (IUPAC): 3-[5-(difluoromethoxy)-1-methyl-3-(trifluoromethyl)pyrazol-4-yl methylsulfonyl]-4,5-dihydro-5,5-dimethylisoxazole
CAS Number: 447399-55-5
Molecular Formula: $C_{12}H_{14}F_5N_3O_4S$
Molecular Weight: 391.3
Lot No.: LP001
Purity: 99.9%
Analytical Methods: HPLC area percent distribution method and moisture analysis

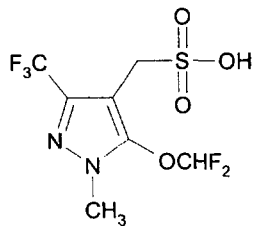
Data of Analysis: November 9, 2007
Date of Synthesis: January 6, 2005
Structure:



Storage Conditions: <30°C
Expiration Date: November 8, 2009

KIH-485 M-1

Chemical Name: (5-difluoromethoxy-1-methyl-3-trifluoromethyl-1*H*-pyrazol-4-yl)-methane sulfonic acid
Molecular Formula: $C_7H_7F_5N_2O_4S$
Molecular Weight: 310.20
Structure:



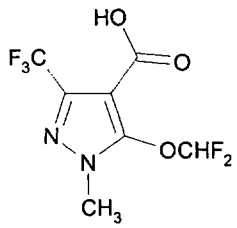
Lot Number: 2
Purity: 97.54%
Method of Analysis: Area percent method via HPLC peak areas
Date of Analysis: November 30, 2007

Storage Condition: 4°C
Expiration Date: November 29, 2009

KIH-485 M-3

Chemical Name: 5-difluoromethoxy-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
Molecular Formula: $C_7H_5F_5N_2O_3$
Molecular Weight: 260.12

Structure:



Lot Number: 4
Purity: 99.60%
Method of Analysis: Area percent method via HPLC peak areas
Date of Analysis: November 30, 2007
Storage Condition: 4°C
Expiration Date: November 29, 2009

Preparation of Stocks and Standards

Primary stock solutions of KIH-485 and its M-1 and M-3 metabolites were prepared in acetonitrile. Solutions were prepared at 1.00 mg a.i./mL (active ingredient/mL) by compensating for the purity of each analyte. Aliquots from each of the primary stock solutions were combined to prepare a secondary stock solution containing KIH-485, M-1 and M-3 at 10.0 µg a.i./mL each in acetonitrile. An aliquot of this combined stock solution was further diluted with acetonitrile to prepare a combined stock solution at 1.00 µg a.i./mL. Working calibration standards in acetone, ranging in concentration from 0.000500 to 0.100 µg a.i./mL, were prepared from the 1.00- and 10.0-µg a.i./mL stocks for the analysis of KIH-485, M-1 and M-3 in validation samples. All stocks solutions were prepared using volumetric flasks, and gas-tight syringes. Stock solutions of KIH-485, M-1 and M-3 were stored under freezer conditions when not in use.

Fortification of Recovery Samples

Samples were prepared by fortification with the 1.00- and 10.0-µg a.i./mL combined stock standard solutions of the KIH-485, M-1 and M-3. Subsamples were fortified at the LOQ and 10x the LOQ.

Water Extraction and Analysis of KIH-485, M-1 and M-3

For each matrix blank and fortified sample, a 5-mL volumetric flask was partially filled with freshwater followed by fortification with the appropriate combined standard solution. The volumetric flasks were brought to volume with freshwater. Aliquots were microfilterfuged as necessary prior to analysis by LC/MS/MS.

Quantitation of KIH-485, M-1 and M-3 by LC/MS/MS

A Hewlett-Packard Model 1100 High Performance Liquid Chromatograph connected to an Applied Biosystems/MDS Sciex API 3000 Triple Quadrupole Mass Spectrometric Detector (LC/MS/MS) was used to analyze samples. An acidified (0.05% formic acid) acetonitrile:water gradient was used.

Quantitation was performed by summing the responses of the primary ion transition and a secondary transition, referred to as the confirmatory ion transition. The ion transitions monitored are summarized below:

Transition	KIH 485	M-1	M-3
Primary	392→229	309→259	259→215
Confirmatory	392→179	309→195	259→165

Specific details of the LC/MS/MS instrumentation and operational parameters are presented in Table 1.

Calculation of Method Recoveries

For each analyte, regression analysis was applied to the chromatographic peak area responses determined in calibration standard solutions versus the respective nominal concentrations of the analyte. Standard curves were generated by plotting this function with analyte concentration (µg a.i./mL) on the abscissa and the respective peak area response on the ordinate. Representative standard curves are presented in Figures 1-3 for KIH-485, M-1 and M-3, respectively. A linear, 1/x weighted, regression analysis was used for quantitating all analytes.

The linear regression equation, derived from regression of peak areas and known nominal concentrations of calibration standard solutions, was expressed as follows:

$$\text{Peak Area} = \text{Slope} \times \text{Concentration} + \text{y-Intercept}$$

Concentrations of analytes in the final solutions of fortified samples were calculated using a rearrangement of the above equation:

$$\text{Concentration} = \frac{\text{Peak Area - y-Intercept}}{\text{Slope}}$$

A representative calculation is presented below consisting of the quantitation of KIH-485 in a freshwater fortified at the target LOQ of 0.0050 mg a.i./L. The same equations and calculations were applied for quantitation of the concentration of M-1 and M-3. Using the results from the weighted linear regression analysis, the equation relating concentration at the instrument and peak area was derived as follows:

Slope = 59,490,200
y-Intercept = 5,218.95
r^2, the coefficient of determination = 0.9943

$$\text{Concentration} = \frac{\text{Peak Area - (-5218.95)}}{59490200}$$

The concentration of KIH-485 in each freshwater sample was determined by substituting the resulting analyte peak area into the above equation. Using the peak area for a 0.0050-mg-a.i./L fortification, Sample Number 267C-117-VMAS-1, the concentration in the final sample solution was calculated as:

$$\text{Concentration} = \frac{321700 - (5218.95)}{59490200} = 0.005320 \text{ µg a.i./mL}$$

The residue concentration (mg a.i./L) for KIH-485 was determined as the product of the solution concentration determined above and the dilution factor for the sample as follows:

$$\text{Concentration in mg a.i./L} = \text{Concentration} \times \frac{\text{(Final Volume)}}{\text{(Initial Volume)}} \times \frac{0.00100 \text{ mg}}{1.000 \text{ µg}}$$

where: Final Volume = 5.0 mL
Initial Sample Mass = 0.00500 L

$$\text{Concentration in mg a.i./L} = 0.005320 \text{ µg a.i./mL} \times \frac{5.00 \text{ mL}}{0.00500 \text{ L}} \times \frac{0.00100 \text{ mg}}{1.000 \text{ µg}}$$

Concentration in mg a.i./L = 0.005320 mg a.i./L

Using the nominal fortification concentration for Sample Number 267C-117-VMAS-1, 0.0050-mg a.i./L, the percent recovery was determined by dividing the concentration of the analyte recovered in the fortified sample by the nominal concentration added as shown below:

$$\text{Recovery (\%)} = \frac{\text{mg a.i./L Found}}{\text{mg a.i./L Added}} \times 100$$

$$\text{Recovery (\%)} = \frac{0.005320 \text{mg a.i./L}}{0.00500 \text{ mg a.i./L}} \times 100$$

Recovery (%) = 106%

Statistical Treatment of Data

Mean recoveries for each analyte were calculated by dividing the sum of the percent recoveries by the total number of fortified samples. The standard deviation and relative standard deviation (coefficient of variation) for the recoveries for each analyte were also determined and reported.

Table 1. LC/MS/MS Instrumentation and Operational Parameters.

Instrumentation	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Applied Biosystems /MDS Sciex API 3000 Triple Quadrupole Mass Spectrometric Detector (LC/MS/MS) and Turbo Ion Spray (TIS) Ion Source
Analytical Column	Michrom Magic AQ C-18; 150 x 2.0 mm, 5-µm particle size
Guard Column	UPCHURCH C-18, 10 mm x 4.3 mm, 5-µm particle size
Mobile Phases	A1: 0.05% Formic acid in reagent-grade water B1: 0.05% Formic acid in methanol Gradient Elution Program: Time (min) / %A1 / %B1 / Flow Rate (µL/min) / Temp (°C) 0.00 / 90.0 / 10.0 / 230 / 40.0 5.00 / 90.0 / 10.0 / 230 / 40.0 20.0 / 0.0 / 100 / 230 / 40.0 24.0 / 0.0 / 100 / 350 / 40.0 25.0 / 90.0 / 10.0 / 230 / 40.0 31.0 / 90.0 / 10.0 / 230 / 40.0
Diverter Valve (Valco)	Time (min) 0.0 divert to waste 14.0 to MS source 25.0 divert to waste
Injection Volume	10 µL
Total Run Time	31 minutes
Period 2	Scan Type/Polarity: MRM/Positive NEB = 12, CUR = 8, CAD = 6, IS = 5000, TEM = 400, DP = 30, FP = 150, EP = 10
KIH-485	Transition 1: (392/229 amu), CE=23, CXP=12 Transition 2: (392/179 amu), CE=45, CXP=10 Retention Time: Approximately 21.7 minutes
Period 1	Scan Type/Polarity: MRM/Negative NEB = 12, CUR = 8, CAD = 6, IS = -4500, TEM = 400, DP = -30, FP = -150-, EP = -10
M-3	Transition 1: (259/215 amu), CE = -12 CXP = -9 Transition 2: (259/165 amu), CE = -20, CXP =-7 Retention Time: Approximately 20.5 minutes
M-1	Transition 1: (309/259 amu), CE = -24, CXP = -11 Transition 2: (309/195 amu), CE = -30, CXP = -9 Retention Time: Approximately 20.4 minutes