PURPOSE

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

EXPERIMENTAL DESIGN

Soil was fortified with KIH-485 and its metabolites M-1 and M-3 at two different concentrations and analyzed according to the methods supplied by the Sponsor. Reagent and matrix blanks (controls) were analyzed concurrently to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Matrix - Origin, Characterization, and Storage

An untreated soil sample representative of that used for growing soybeans was collected and characterized by Agvise laboratories and provided to Wildlife International, Ltd. The soil was received at Wildlife International, Ltd. on October 17, 2006. Upon receipt at Wildlife International, Ltd., the soil sample (identified as MSL-PF) was stored refrigerated. The soil, classified as a sandy clay loam, was characterized as follows: 61% sand, 21% clay, 18% silt, pH 6.8, 3.8% organic matter, 16.0 meq/100g CEC and 0.96 g/cc bulk density. A GLP Soil Characterization Report was provided by Agvise laboratories and is presented in Appendix III.

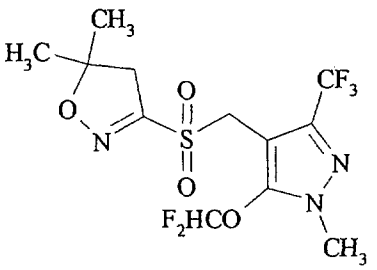
Test Substances

Test substances KIH-485, KIH-485 M-1 and KIH-485 M-3 were received from LSG Corporation on April 19, 2006. The test substances were assigned Wildlife International, Ltd. Identification Numbers 7585, 7586 and 7587, respectively. Test substance KIH-485 was stored under ambient conditions and the metabolites M-1 and M-3 were stored under refrigerated conditions in darkness. Certificates of Analysis were received with each test substance and provided the following information with the exception of the structure for KIH-485. The structure of KIH-485 was obtained from additional information supplied by Kumiai Chemical Industry Co., Ltd.

KIH-485
Chemical Name (IUPAC): 3-[5-(difluoromethoxy)-1-methyl-3-(trifluoromethyl)pyrazol-4-yl methylsulfonyl]-4,5-dihydro-5,5-dimethyl-1,2-oxazole
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

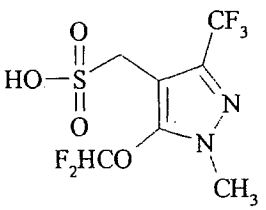
Data of Analysis: September 30, 2005
Date of Synthesis: January 6, 2005

Structure:



Storage Conditions: <30°C
Expiry Date: September 29, 2007

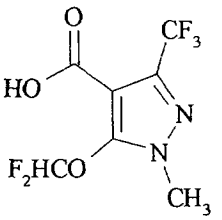
KIH-485 M-1
Chemical Name: (5-difluoromethoxy-1-methyl-3-trifluoromethyl-1*H*-pyrazol-4-yl)-methane sulfonic acid
CAS Number: Not Given
Molecular Formula: $C_7H_7F_5N_2O_4S$
Molecular Weight: 310.20
Lot Number: 2
Purity: 97.8%
Method of Analysis: Area percent method via HPLC peak areas
Date of Analysis: December 20, 2005
Structure:



Storage Condition: 4°C
Expiration Date: December 19, 2007

KIH-485 M-3
Chemical Name: 5-difluoromethoxy-1-methyl-3-trifluoromethyl-1*H*-pyrazole-4-carboxylic acid
CAS Number: Not Given
Molecular Formula: $C_7H_5F_5N_2O_3$
Molecular Weight: 260.12
Lot Number: 4
Purity: 99.6%
Method of Analysis: Area percent method via HPLC peak areas
Date of Analysis: December 19, 2005

Structure:



Storage Condition: 4°C
Expiration Date: December 18, 2007

Preparation of Stocks and Standards

Primary stock solutions of KIH-485 and the M-3 metabolite were prepared in acetonitrile at 1000 µg a.i./mL. Aliquots from each of the primary stock solutions were combined to prepare a secondary stock solution containing KIH-485 and the M-3 metabolite at 10.0 µg a.i./mL in acetonitrile. The combined stock solution was further diluted in acetonitrile to prepare combined stock solutions at 1.00 and 0.500 µg a.i./mL in acetonitrile. An additional stock solution was prepared in acetonitrile by diluting an aliquot from the 0.500 µg a.i./mL stock to a concentration of 0.100 µg a.i./mL. Working calibration standards in acetone, ranging in concentration from 0.00100 to 0.500 mg a.i./mL, were prepared from the 0.500 and 10.0 µg a.i./mL stocks for the analysis of KIH-485 and M-3 in validation samples. All stocks solutions were prepared using volumetric flasks, pipettes, and/or gas-tight syringes. The stock solutions were stored under freezer conditions when not in use.

A primary stock solution of the M-1 metabolite was prepared in acetonitrile at 1000 µg a.i./mL. Aliquots from the primary stock solution were diluted in acetonitrile to prepare stock solutions at 1.00 and 0.500 µg a.i./mL and the 1.00 µg a.i./mL stock solution serially diluted in acetonitrile to prepare a stock solution at 0.100 µg a.i./mL. Working calibration standards in 70% acetonitrile: 30% water, ranging in concentration from 0.00500 to 0.500 mg a.i./L, were prepared from the 0.500 and 10.0 µg a.i./mL stocks for the analysis of M-1 in validation samples. All stocks solutions were prepared using volumetric flasks, pipettes, and/or gas-tight syringes. The stock solutions were stored under freezer conditions when not in use.

Chromatography Evaluation

Prior to performing the definitive method trials, Wildlife International, Ltd. analyzed calibration standards of each analyte and demonstrated that they could produce acceptable chromatographic data.

Fortification of Recovery Samples

Fortification samples were prepared as described in the analytical method. The soil matrix recovery samples were fortified with either KIH-485 and the M-3 metabolite or the M-1 metabolite at 0.002 mg a.i./Kg (LOQ) and 0.02 mg a.i./Kg (10x LOQ), as specified in the protocol.

Sample Extraction and Analysis

Once the soil matrix recovery samples were fortified with either KIH-485 and M-3 or M-1, they were extracted as per the respective procedures outlined in the Sponsor supplied methodologies (Appendices II-III). As details on the different methods may be found in the Appendices, only a brief description is provided here. The soil extraction methodology for KIH-485 and M-3 incorporated an acetone/water extraction, filtration and solvent reduction steps, a liquid-liquid extraction with ethyl acetate for KIH-485, and a second liquid-liquid extraction following acidification for the M-3 metabolite. The KIH-485 extract was subsequently concentrated and purified using a column cleanup consisting of two SPE stationary phases (DSC-NH2 and ENVI CARB). The M-3 extract was concentrated and then combined back with the KIH-485 extract for a final concentration of the combined extract. Samples were reconstituted with acetone and analyzed by HPLC/MS/MS.

The soil extraction methodology for the M-1 metabolite incorporated a water extraction, filtration and concentration of the aqueous extract followed by purification using a column cleanup consisting of a single SPE stationary phase (HLB) followed by a concentration step. Samples were reconstituted with 30% acetonitrile: 70% water (v:v) and analyzed by HPLC/MS/MS.

A Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a PE Sciex API 3000 Triple Quadrupole Mass Spectrometric Detector (LC/MS/MS) was used to analyze samples. Further details of the LC/MS/MS instrumentation and operational parameters are presented in Table 1.

Calculation of Percent Recovery

Standard curves were prepared by plotting the analyte concentration (µg a.i./mL) on the abscissa and the respective peak area response on the ordinate as shown in Figures 1-3. A quadratic (1/x weighted) regression analysis was used for quantitating KIH-485 and M-3. A linear regression analysis was used for quantitating M-1.

For KIH-485 and M-3 metabolite, the regression analysis was applied to the data to determine the equation with respect to the abscissa. For purposes of this calculation section, data for KIH-485 only will be used. The same equations and calculations were applied for the quantitation of the M-3 metabolite. An example calculation for KIH-485 is shown below. The curve was weighted 1/x with respect to concentration and expressed as a quadratic function as follows:

$$y = Ax^2 + Bx + C$$

where y = instrumental peak area response of concentration x of KIH-485 in mg a.i./Kg
A = quadratic coefficient
B = linear coefficient
C =constant coefficient (Y_intercept)

Concentrations of KIH-485 in the samples were determined by substituting peak area responses of the samples into the applicable rearranged regression equation, corrected for dilution factor, as follows:

$$\text{KIH-485 (mg a.i./kg)} = \text{Dilution Factor} \times \frac{-B + \sqrt{B^2 - [4 \times A \times (C - \text{Peak Area})]}}{2 \times A}$$

Data used for quantitation of KIH-485 in a 0.0200 mg a.i./kg fortification (Sample Number 267C-102-VMAS-1 are summarized below:

Peak area = 131750
Constant Coefficient (C) = -2155.15
Linear Coefficient (B) = 9543100
Quadratic Coefficient (A) = -2523250
Dilution Factor ($V_{final}/M_{initial}$) = 2.00 mL /15.0 g = 0.133

$$\text{KIH-485 (mg a.i./kg)} = 0.133 \times \frac{-9543100 + \sqrt{(9543100)^2 - [(4 \times (-2523250)) \times (-2155.15 - 131750)]}}{2 \times (-2523250)}$$

KIH-485 (mg a.i./kg) = 0.00187

The measured concentration was compared to the nominal concentrations as follows:

$$\text{Percent of nominal concentration} = \frac{\text{KIH-485 in sample (mg a.i./kg)}}{\text{KIH-485 nominal concentration (mg a.i./kg)}} \times 100$$

$$= \frac{0.00187 \text{ mg/L}}{0.002 \text{ mg/L}} \times 100$$

$$= 93.7\%$$

For M-1 metabolite, the regression analysis was applied to the data to determine the equation with respect to the abscissa as shown below.

$$PA = mC + b$$

Where: PA = peak area
m = slope
C = concentration
b = y-intercept

Concentrations of analyte in the final solutions of M-1 samples were calculated using a rearrangement of the above equation:

$$C = \frac{PA - b}{m}$$

Using the results from the linear regression analysis with the data from Figure 2:

$$C = \frac{PA - 7238.94}{10918100}$$

The net concentration of M-1 in each corresponding recovery sample was determined by substituting the resulting analyte peak area into the above equation and solving for the concentration. Using the data for a 0.0200 mg a.i./kg fortification (Sample Number 267C-102-VMAS-17), the concentration in the final sample solution was calculated as:

$$C = \frac{1597800 - 7238.94}{10918100} = 0.14568 \text{ mg a.i./Kg}$$

The residue concentration (mg a.i./Kg) for the M-1 metabolite in the fortified recovery sample was determined as the product of the determined concentration from above and the dilution factor for the sample as follows:

$$\text{Concentration in mg a.i./Kg} = (C) \times \frac{(V_f)}{(M_i)}$$

Where: C = Concentration (mg a.i./kg) as determined above
V_f = Final volume (2.00 mL)
M_i = Initial sample mass (15.0 g)

Using the data from the 0.0200 mg a.i./kg fortification sample (267C-102-VMAS-17), the concentration of the M-1 metabolite in soil was calculated as:

$$\text{Concentration in mg a.i./kg} = 0.14568 \times \frac{2.00}{15.0}$$

$$\text{Concentration in mg a.i./kg} = 0.14568 \times 0.133$$

$$\text{Concentration in mg a.i./kg} = 0.01938 \text{ mg a.i./kg}$$

The percent recovery was determined by dividing the concentration of the analyte recovered in the fortified sample by the theoretical concentration added as shown below:

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

For the above 0.0200 mg a.i./Kg fortified sample, the percent recovery for M-1 was calculated as:

$$\text{Recovery (\%)} = \frac{0.01938 \text{ mg a.i./Kg Found}}{0.0200 \text{ mg a.i./Kg Added}} \times 100$$

$$\text{Recovery (\%)} = 96.9$$

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

Instrumentation:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a PE 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, 5µm
Guard Column	Thermo EC Javalin Betasil C-18 (10mm x 2 mm I.D.)
Mobile Phases:	A2: 0.05% Formic acid in reagent grade water B2: Acetonitrile with 0.05% formic acid Gradient Elution Program: Time (min) %A2 %B2 Flow Rate (µL/min) Temp (°C) 0.00 95 5.0 250 40.0 0.01 95 5.0 250 40.0 20.0 0.0 100 250 40.0 25.0 0.0 100 250 40.0 27.0 95 5.0 250 40.0 35.0 95 5.0 250 40.0
Injection Volume:	10 µL
Total Run Time:	35 minutes
Mass Spectrometer Conditions: KIH-485	Scan Type/Polarity: MRM/Positive Transition 1: (392/229 amu), CE=23, CXP=12 Transition 2: (392/179 amu), CE=45, CXP=10 NEB=12, CUR=8, CAD=6, IS=5500, TEM=450, DP=56, FP=350-, EP=10 Retention Time: Approximately 17.8 min.
Mass Spectrometer Conditions: M-3 Metabolite	Scan Type/Polarity: MRM/Negative Transition 1: (259/215 amu), DP=-26, CE=-12, CXP=-9 Transition 2: (259/165 amu), DP=-21, CE=-20, CXP=-7 NEB=10, CUR=8, CAD=4, IS=-4500, TEM=450, FP=-250-, EP=-10 Retention Time: Approximately 14.6 min.
Mass Spectrometer Conditions: M-1 Metabolite	Scan Type/Polarity: MRM/Negative Transition 1: (309/259 amu), CE=-24, CXP=-11 Transition 2: (309/195 amu), CE=-30, CXP=-9 NEB=12, CUR=8, CAD=6, IS=-4500, TEM=450, DP=-51, FP=-350-, EP=-10 Retention Time: Approximately 11.9 min.