Determination of Residues of AKD 2023 and Its Major Metabolite Rl (Hydroxy AKD·2023) in Drinking Water, Ground Water and Surface Water

1. INTRODUCTION

This summary level document modifies the original validated method by incorporating minor method changes employed during the independent laboratory validation.

The original method: "Development and Validation of Methodology for the Determination of AKD-2023 and its Major metabolite R1 (Hydroxy AKD-2023) in Drinking Water, Ground Water, and Surface Water" (Appendix II of MRID46182602) was validated by Huntingdon Life Sciences Limited, Woolley Road, Alconbury, Huntington, Cambridgeshire, PE28 4HS, England.

The Independent laboratory Validation "Independent Laboratory Validation for the Determination of Acequinocyl, Acequinocyl-OH, and AKM-18 in Water and Soil (MRID 46182602) was performed by Pyxant Labs Inc, 4720 Forge Road, Suite 108, Colorado Springs, CO 80907

Reasons for Revision:

During the course of the ILV, the following modifications and additions were made to the sample preparation procedure of the Huntingdon Life Sciences Method AGK/076:

1) Prior to any standard or sample preparation, all glassware was silylated.
2) All stock. solutions and final working solutions were kept in amber glass bottles wrapped in aluminum foil
3) (Lights in the laboratory were turned off during the preparation of standards, fortification, extraction, partition, and throughout the rotary evaporation process because of the sensitivity of acequinocyl and acequinocyl-OH to light
4) The temperature on the rotary evaporator was kept at 27°C, and the pressure was approximately 15 inches of Hg. The ending pressure was 18 inches of Hg, and the evaporation time was approximately 20 minutes.
5) After the first rotary evaporation step, the 250 mL round bottomed flask was rinsed twice with approximately 3 mL hexane, and sonicated after each rinse.
6) In the final step of the method, the solvent was removed under nitrogen evaporation at ambient temperature and not in a 30°C water bath to reduce sample degradation.
7) The mass spectrometric and chromatographic procedures used in the Huntington method were also modified. The method specifies ions to be monitored, which are attributable to the molecular ions of the compounds (M+). This is a limitation because typically, when using electrospray atmospheric pressure mass spectrometry in the positive ion mode, the major ions observed are due to the protonated forms of the compounds (M+H)+. Therefore, ions monitored are one mass unit greater than the molecular weight of the compound. Molecular ion peaks (M+) can be observed, but are

instrument dependent and may not be observed on other instruments, even if they are of the same manufacturer and model. Huntington Labs acknowledged the problem, but indicated that in their studies, the molecular ions were of greater intensity and was their choice for the method.

For the independent laboratory, the ions of greater intensity were the more usual protonated ion forms; the molecular ions (M+) were not observed. Therefore, the independent laboratory adapted the mass spectrometric and chromatographic conditions utilized for the same compounds in a soil analysis method developed by Morse Laboratories (Morse Method 136, Appendix 1 of MRID 46182602.) These procedures were suitable for the operation of the instruments at the independent laboratory.

These minor modifications are not considered significant and will have no effect on the quality of the data.

2. MATERIALS AND METHODS

2.1. Test/Reference Substance

The test/reference substances used in this study are described as follows:

Test/Reference Substance:	Acequinocyl
Chemical Name:	3-Dodecyl-1,4-dihydro-1,4-dioxo- 2-naphthyl acetate
CAS #:	57960-19-7
Purity:	97.07%
Lot #:	AK23981/S
Expiration Date:	June 3, 2005

Test/Reference Substance:	Hydroxy-Acequinocyl
Chemical Name:	2-dodecyl-3-hydroxy-1,4-naphthoquinone
CAS #:	57960-31-3
Purity:	95.87%
Lot #:	AK23981/M5
Expiration Date:	June 4, 2005

2.2. Reagents and Materials

The following is a list of the reagents prepared and the materials used for the current study:

Reagents

- Acetonitrile
- Acetone
- Chem-Solv® (Mallinckrodt Chemicals)
- Dimethyldichlorosilane (DMDCS)
- Formic Acid
- Hexane
- Methanol
- Sodium Chloride
- Sodium Sulfate
- Water, UHP
- 5% (v/v) dimethyldichlorosilane in hexane: Add 95 mL hexane to a glass stoppered container. Slowly add 5.00 mL DMDCS, stopper, and invert to mix.
- 0.4% formic acid in water: Add 2.00 mL formic acid to a 500 mL volumetric flask, bring to volume with UHP water and mix.
- Acetone/Acetonitrile/0.4% aqueous formic acid (2/2/1, v/v/v): Add 80 mL acetone, 80 mL acetonitrile and 40 mL 0.4% aqueous formic acid to a 250 mL volumetric flask and mix. Prepare weekly.
- 0.1% Formic Acid in Methanol: Add about 100 mL methanol to a 1 L volumetric flask. Add 1.00 mL formic acid; fill to volume with methanol, and mix.
- 0.1% Formic Acid in Water: Add about 100 mL water to a 1 L volumetric flask. Add 1.00 mL formic acid; fill to volume with water, and mix.

Materials

- Silylated Glass Wool
- Plastic powder funnels
- Polypropylene pipettes
- 50 mL volumetric flasks
- 1L separatory funnel

2.3. Analytical Instrumentation and Equipment

The independent laboratory established the method parameters prior to initiation of the first sample trial by determining the retention times of the analyte and computing the instrument detection limits using analytical standards. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

Instrumentation:

Agilent Model 1100 Autosampler	S/N DE21001902
Agilent Model 1100 Binary Pump	S/N DE14910448
Agilent Model 1100 Degasser	S/N JP13203157
Valco 10 Port Switching Valve	S/N EM2C05582
PE SCIEX API 3000 HPLC/MS-MS	S/N D10590207
Phenomenex Luna Phenyl Hexyl, 6 x 150 mm, 3µm column (groundwater analysis)	S/N 208752-1
Phenomenex Luna C_8 (2) 2 x 150 mm, 3 µm column (soil analysis)	S/N 207129-4

Operating Parameters:

Typical Chromatographic Conditions, Groundwater Analysis

Column Temperature:	35°C
Injection Volume:	50 µL
Run Time:	18 minutes
Mobile Phase:	A – 0.1% Formic Acid in Water B – 0.1% Formic Acid in Methanol
Flow Rate:	0.9 mL/min

Gradient:

Time, min	A%	B%
0-0.4	45	55
8.0	10	90
12.0-15.5	5	95
15.6-18.0	45	55

Typical Mass Spectrometer Parameters

Interface:	TurboIonSpray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – Unit, Q3 – Low
Curtain Gas:	10
Nebulizer Gas:	9
Collision Gas:	7
Temperature:	400°C
Turbo Gas:	8000 mL/min
Ion Spray Voltage:	4500.00 V

Scan Function:
Transitions Monitored:

Compound	*m/z* Q1	*m/z* Q3	Time (ms)	Collision Energy (V)	Declustering Potential (V)	Focusing Potential (V)
Acequinocyl	385	189	200	34	61	220
Acequinocyl-OH	343	189	200	30	66	220

Other Equipment
Balance, Analytical Sartorius, Model AC120S, S/N 20103137
Microbalance, Cahn, Model C-34, S/N C1066/C2251
Balance, Pan Sartorius, Model BA2100S, S/N 20303446
SPE Vacuum Manifold System, Burdick and Jackson, 24 port
Rotary Evaporator, Buchi, Model 011, equipped with B-461 water bath (S/N 143516)
Rotary Evaporator, Buchi, Model 011, equipped with B-461 water bath (S/N 1158505)
N-Evaporator, Organomation, Model 112, S/N 3455 and S/N 50266 (water bath S/N only)
Vortex Mixer, S/P Vortex Mixer Jr., Model S8225-1, S/N 001297
Ultrasonic Cleaner, Mettler, Model ME 4.6, S/N 85M16531

Some instrument and chromatographic parameters were adjusted, as provided in the protocol and the method, to match the performance of the validation method. Further details of these adjustments are discussed in Section 3.5, Groundwater Sample Preparation, and the Reasons for Revision Section. Method performance was not compromised by these minor modifications.

2.4. Standard and Fortification Solution Preparation

Stock solutions of acequinocyl, and hydroxy-acequinocyl, were prepared by accurately weighing approximately 10 mg of each analytical standard into a 50 mL volumetric flask. Acequinocyl was brought to volume in acetonitrile and acequinocyl-OH was brought to volume in acetone, to make nominal 200 µg/mL stock solutions. The stock solutions were stored refrigerated (2°C to 8°C) in the dark when not in use.

Seven mixed calibration standards for were prepared from the stock solutions for each test/reference substance by serial dilution with acetone/acetonitrile/0.4% aqueous formic acid (2/2/1, v/v/v). Standard concentrations for the analysis were 10, 30, 50, 70, 100, 250, and 500 µg/L. Individual fortification solutions of each test/reference substance were prepared by serial dilution from the stock at 0.1 and 1 µg/mL for groundwater by diluting with acetonitrile. Calibration and fortification standards were stored refrigerated (2°C to 8°C) in the dark when not in use

2.5. Groundwater Sample Preparation

Groundwater sample preparation was performed as in the "Method, Procedures" section of the Huntingdon Life Sciences method AGK/076 with the exception of the method modifications listed in Reasons for Revision Section. Prior to any standard or sample preparation, all glassware was silylated. Further, all stock solutions and final working solutions were kept in amber glass bottles wrapped in aluminum foil. Lights in the laboratory were turned off during

the preparation of standards, fortification, extraction, partition, and throughout the rotary evaporation process because of the sensitivity of acequinocyl and acequinocyl-OH to light.

An aliquot (500 mL) of groundwater was transferred to a separatory funnel, and mixed standard solutions were added as follows:

- 0.5 mL of 0.1 µg/mL standard added to 0.5 L of groundwater to fortify at 0.1 µg/L (LOQ)
- 0.5 mL of 1.0 µg/mL standard added to 0.5 L of groundwater to fortify at 1.0 µg/L (10X LOQ)

Approximately 20 g of sodium chloride and 50 mL of hexane were added and the sample was shaken vigorously. The phases were allowed to separate and the upper hexane phase was passed through a funnel containing sodium sulfate into a 250 mL round-bottomed flask. The groundwater was reextracted with another 50 mL hexane, and the extracts were combined in the round-bottomed flask. The sodium sulfate was rinsed with approximately 40 mL hexane and this was combined with the other extracts in the round-bottomed flask. The extract was rotary evaporated to approximately 3 mL at <30°C and the sample was transferred with a plastic pipette tip into a 13 x 100 mm test tube. The 250 mL flask was rinsed twice with 3 mL hexane, sonicating for approximately 45 seconds after each rinse. The solvent was evaporated to approximately 3 mL using an N-Evap nitrogen evaporator at <30°C. The remaining volume was removed just to dryness in the N-Evap nitrogen evaporator at ambient temperature. The sample was reconstituted in 1.00 mL acetonitrile/water/formic acid (85/15/0.2, v/v/v) and sonicated prior to LC/MS/MS analysis.

2.6. Sample Analysis

Samples were analyzed using the parameters listed in Section 3.3, Analytical Instrumentation and Equipment. Chromatographic conditions and mass spectrometric parameters were adjusted from the original conditions in order to optimize peak shape and response.

Seven calibration standards were analyzed with the groundwater sample set over the range of 10 µg/L to 500 µg/L. An analysis set consisted of 2 UTCs, 2 UTCs fortified at the LOQ, 2 UTCs fortified at 10X LOQ, and a reagent blank.

2.7. Method Modifications

Modifications of the original method are discussed in the Introduction under the "Reasons for Revision" heading. These minor modifications are not considered significant and will have no effect on the quality of the data.

2.8. Example Calculations

The calculations of the percent recovery of acequinocyl and acequinocyl-OH from well water were performed as described in the "Calculation of results" section of the Huntingdon Life Sciences method. Calibration standards (10, 30, 50, 70, 100, 250, and 500 µg/L) were analyzed with each sample set. A best fit calibration curve using linear regression with $1/x^2$ weighting (acequinocyl) and linear regression with 1/x weighting (acequinocyl-OH) was generated for calibration standards using the respective peak area responses versus the concentration of the

calibration standards. Concentrations of analyte in the samples were determined by substituting the peak area responses into the linear with 1/x weighting regression equation as shown below:

$$y = mx + b$$

$$x = \frac{y - b}{m}$$

Where x is the concentration found in µg/L and y is the peak area response. The slope, m, and intercept, b, were calculated by the Excel software program and independently verified using hand calculations.

For example, using the acequinocyl data from 1486-38, control well water fortified at 1 µg/L (Figure 36), where the observed area count is 5.71589E+03.

$$\text{µg/L from curve} = \frac{5.71589E+03 - (-582.14)}{78.26} = 80.5 \text{ µg/L}$$

$$\text{Sample µg/L} = \frac{\text{µg/L from curve x Dilution Factor}}{500 \text{ mL/mL}}$$

$$\text{Sample µg/L} = \frac{80.5 \text{ µg/L x } 5}{500 \text{ mL/mL}}$$

$$\text{Sample µg/L} = 0.805$$

Percent recovery is calculated by dividing the concentration by the fortification amount, in this case, 1 µg/L:

$$\text{Recovery} = \left(\frac{\text{Conc. Found}}{\text{Conc. Added}}\right) \text{x } 100\%$$

$$\text{Recovery} = \left(\frac{0.805 \text{ µg/L}}{1.00 \text{ µg/L}}\right) \text{ x } 100\%$$

$$\text{Recovery} = 80.5\%$$

2.9. Statistical Treatment of Data

The mean recoveries for the samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program which divides the sum of the selected cells by the number of determinations. The standard deviations were calculated using the "STDEV" function of the same spreadsheet program which sums the squares of the individual deviations from the mean, divided by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation (% RSD) is calculated by dividing the standard deviation by the mean, then multiplying by 100.

APPENDIX I

Silylation of Glassware

Silylation is a process used to chemically treat glassware or other products in order to prevent or minimize binding of analyte residues to the glass surface.

Caution: **Do not allow dimethyldichlorosilane (DMDCS) to come in contact with water. Chlorine gas or hydrogen chloride gas will be produced.**

This procedure must be conducted inside an efficient fume hood. Heavy latex gloves must be worn.

1. Completely remove all traces of residues from the glassware using concentrated Chem-Solv as the cleaning agent. After cleaning, rinse the glassware with copious amounts of deionized water until the rinseate is no longer basic. Upon drying, the glassware can then be silylated as described below.

2. Pour a small amount of the 5% DMDCS solution into the glassware to be treated. Stopper bulk container. Rotate the glassware to thoroughly coat the inside surfaces. Pour excess solution into the next piece of glassware to be treated.

   Note: Moisture in the air tends to inactivate this reagent. To insure maximum activity of the silylating agent during the coating process, limit the exposure (to the atmosphere) of the silylating agent to approximately 5 minutes.

3. Allow the treated glassware to dry (approximately 20 minutes). Rinse thoroughly with hexane, then reagent acetone. Again allow to dry.

4. Glassware is now ready for use.

   Notes: Any glassware that is cleaned with a brush after it has been silylated must be resilylated.

   Store pure DMDCS at room temperature.

   5% solutions of DMDCS in hexane are stable for 5 days when stored well stoppered at room temperature. Choose a storage container with minimum air space above the surface of the solution.

Silylation of Glass Wool:
In a fume hood, while wearing heavy latex gloves, place a quantity of glass wool into a large wide mouth glass jar with Teflon®-lined lid (e.g., ~400 mL capacity). Saturate the glass wool with the silylating solution (using approximately 200 mL). **To assure that thorough saturation has**

occurred, stir to thoroughly coat the glass wool in the glass jar with a sturdy glass rod. Cap tightly and allow to stand for approximately 20 minutes.

Decant the silylating solution from the glass jar and while wearing latex gloves, squeeze the excess silylating solution from the glass wool. Pull the wet glass wool apart to aid in drying. Place on clean paper towels in a hood to thoroughly dry. This drying may require as long as 4-8 hours.

When completely dry, return the silylated glass wool into a glass jar and rinse thoroughly three times with hexane followed by three rinses with acetone.

Allow the glass wool to dry on clean paper towels. Store the dry, silylated glass wool in a covered glass container. **The washed and dried silylated glass wool may be used for up to one month.**