INTRODUCTION

The objective of this study was to independently validate the PTRL West residue method 1669W for the determination of kasugamycin in soil. The independent validation (ILV) was conducted using untreated control soil, which is representative for the intent of the method. With modifications, the method was found to be suitable for the determination of kasugamycin and kasugamycinic acid in soil over the concentration range 0.01 µg/g to 0.10 µg/g with a validated limit of quantitation (LOQ) of 0.01 µg/g.

This independent laboratory validation was conducted to satisfy the requirements of the European Council Directive 91/414/EEC, as amended by European Commission Directive 96/46/EC, and the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 (2, 3, 4). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 850.7100 (5), and PR Notice 96-1 (6). This validation report presents the results of the independent laboratory validation for kasugamycin and kasugamycinic acid in soil.

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing the soil samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between the Sponsor or PTRL West and the Study Director or analyst. Throughout the conduct of the study, any communications between the Sponsor or PTRL West and the Study Director and/or the analyst were logged for inclusion in the report. No one from the Sponsor or PTRL West was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Sample Receipt, Labeling and Storage

Two shipments of untreated control soil samples were shipped from PTRL West and received frozen and in good condition on November 1, 2010 and February 3, 2011, respectively. Upon receipt, the shipments were assigned unique master logbook (MLB) numbers 22347134 and 22347260, respectively, and stored frozen (approximately -20°C).

Preparation of Solutions and Standards

The analytical reference standards/test substances utilized during the independent laboratory method validation are summarized below. The reference standards were received from the Sponsor and stored as indicated. The Certificates of Analysis are included in Appendix A.

The kasugamycinic acid reference standard (lot number 01), was initially certified by Hokko Chemical Industry Co., LTD at 100% purity (Appendix A). Following the completion of the analytical portion of this study, a new COA for lot number 01 was generated by PTRL West (7) in which the purity is reported as 86.7% (also in Appendix A).

Standard	**Percent Purity**	**Recertification Date**	**Lot Number**	**Storage Conditions**
Kasugamycin Hydrochloride Hydrate (KSM)	99.4% (87.3% free base)	December 16, 2011	VII-I	Frozen
Kasugamycinic Acid (KSMA)	100%	November 15, 2013	01	Frozen
	86.7%	January 2013		

For clarity, the kasugamycinic acid concentration of standards and solutions calculated using both purity values are shown below.

Standard solutions and calibration standard solutions were prepared as described below and stored refrigerated (approximately 2-8°C) when not in use.

Stock solutions were prepared as follows:

Stock Solution	Solution Lot Number	Weight [mg]	Dissolve In	Obtain [µg/mL]*	
Kasugamycin	N707P06-A	19.943	25.0 mL (100.19 mL methanol + 43 mL 5% aqueous ammonium acetate)	696.4	
	N707P06-B	10.924	10.0 mL water	953.7	
Kasugamycinic Acid	N707P07-A	10.341	10.0 mL water	1034	***896.6***
	N707P07-B	10.163	10.0 mL water	1016	***881.1***

*Resulting concentrations after correcting for purity. Kasugamycinic acid concentrations corrected with recertified purity value of 86.7% are shown in ***bold italics***.

Mixed intermediate and fortification solutions were prepared in water:

From Solution Lot Numbers	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain [µg/mL]*		Final Solution Lot Number
N707P07-B	1016	246	25.0	10.0	***8.67***	N707P08-A
N707P06-B	953.7	263		10.0		
N707P07-B	1016	246	25.0	10.0	***8.67***	N707P14-A
N707P06-B	953.7	263		10.0		
N707P08-A	10.0	2500	25.0	1.00	***0.867***	N707P08-B
N707P08-B	1.00	2500	25.0	0.100	***0.0867***	N707P09-A
N707P14-A	10.0	2500	25.0	1.00	***0.867***	N707P15-A
N707P15-A	1.00	2500	25.0	0.100	***0.0867***	N707P15-B

* Concentrations corrected with kasugamycinic acid recertified purity value of 86.7% are shown in ***bold italics***.

Mixed calibration standards were prepared in water:

From Solution Lot Numbers	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain [µg/mL]*		Final Solution Lot Number
N707P08-A	10.0	500	25.0	0.200	***0.173***	N707P09-B
N707P08-A	10.0	250	25.0	0.100	***0.0867***	N707P10-A
N707P08-A	10.0	125	25.0	0.0500	***0.0434***	N707P10-B
N707P08-B	1.00	625	25.0	0.0250	***0.0217***	N707P11-A
N707P08-B	1.00	250	25.0	0.0100	***0.00867***	N707P11-B
N707P08-B	1.00	125	25.0	0.00500	***0.00434***	N707P12-A
N707P09-A	0.100	625	25.0	0.00250	***0.00217***	N707P12-B
N707P09-A	0.100	250	25.0	0.00100	***0.000867***	N707P13-A

* Concentrations corrected with kasugamycinic acid recertified purity value of 86.7% are shown in ***bold italics***.

Additional mixed calibration standards were prepared in water:

From Solution Lot Numbers	Conc. [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain [µg/mL]*		Final Solution Lot Number
N707P14-A	10.0	250	25.0	0.100	***0.0867***	N707P16-G
N707P14-A	10.0	125	25.0	0.0500	***0.0434***	N707P16-F
N707P15-A	1.00	625	25.0	0.0250	***0.0217***	N707P16-E
N707P15-A	1.00	250	25.0	0.0100	***0.00867***	N707P16-D
N707P15-A	1.00	125	25.0	0.00500	***0.00434***	N707P16-C
N707P15-B	0.100	625	25.0	0.00250	***0.00217***	N707P16-B
N707P15-B	0.100	250	25.0	0.00100	***0.000867***	N707P13-A

* Concentrations corrected with kasugamycinic acid recertified purity value of 86.7% are shown in ***bold italics***.

Fortification of Recovery Samples

The ILV trial of the method was performed for kasugamycin and kasugamycinic acid in soil. The trial was comprised of one batch for each analyte, which consisted of the following samples:

1 (one) reagent blank (containing no matrix or analyte)

1 (one) reagent blank spike at the LOQ level

2 (two) unfortified control samples

5 (five) control samples fortified at 0.01 μg/g, the LOQ of the method

5 (five) control samples fortified at 0.10 μg/g, or 10×LOQ

For preparation of recovery control specimens, appropriate volumes of the fortification standards were added as indicated below:

Specimen Portion	Nominal Target Fortification Level [μg/g]	Actual Nominal Fortification Level for Kasugamycinic Acid [μg/g]*	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [μg/mL]
50 g	0.0100	0.00867	0.500	1.00
	0.100	0.867	0.500	10.0

* Corrected with recertified purity value of 86.7%

Sample Analysis

The ILV trial was conducted as described in the PTRL West residue analytical method 1669W (1), with modifications.

For the analysis of kasugamycin, the soil samples (50 g) were exhaustively extracted by shaking with acidified HPLC water and then the extracts were centrifuged, and then combined. An aliquot of the sample extracts were either analyzed directly by HPLC employing mass spectrometric detection (LC/MS/MS) or diluted as needed; the remaining extract aliquots were reserved.

For the analysis of kasugamycinic acid, the soil samples (50 g) were exhaustively extracted by shaking with basified HPLC water and then the extracts were centrifuged, and then combined. The sample extracts were combined with the reserved kasugamycin extracts and concentrated with methanol and using rotary evaporation. The sample residue was diluted to 1.25 mL with HPLC water with 0.375 mL 20 mM EDTA, microcentrifuged, and then diluted as needed or analyzed for kasugamycinic acid by LC/MS/MS.

For more specific details, refer to the analytical method (1) and the method modifications described in the Results and Discussion section.

Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the extraction and chromatographic instrumentation supplied by the method. These preliminary studies established that adequate retention times of the analytes and detector sensitivity could be achieved. The prepared standards that were used were also used throughout the remainder of the study. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

Instrumentation, Kasugamycin

Typical HPLC Conditions

Instrument: Waters Acquity (System 5)
Column: SeQuant Zic Hilic, 5 µm, 200A, 4.6 × 15 mm
Temperature: Ambient
Injection Volume: 20 µL
Run Time: 22 minutes
Mobile Phase: A: Water with 0.5% formic acid
B: Acetonitrile with 0.5% formic acid

Gradient:

Time (min)	% A	% B	Flow Rate (µL/min)
0.00	0	100	600
5.00	0	100	600
10.00	100	0	600
15.00	100	0	600
16.00	0	100	600
17.00	0	100	900
22.00	0	100	900

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 4000 Mass Spectrometer
Detector Mode: Positive-ion electrospray
Source Temperature: 450°C
Approximate Retention Time: 11.6 min

Ions Monitored:

	Transition	Declustering Potential V	Collision Energy eV	Dwell Time ms	Cell Exit Potential CXP
Kasugamycin	380 → 200	40	18	200	6
	380 → 112	47	30	200	9

Instrumentation, Kasugamycinic Acid

Typical HPLC Conditions

Instrument: Agilent 1100 (System 5)

Column: Luna NH2, 5 µm, 4.6 × 250 mm

Temperature: Ambient

Injection Volume: 50 µL

Run Time: 26 minutes

Mobile Phase: A: 96/2/2 acetonitrile/water/0.5 mM ammonium formate buffer (pH 3)

B: 2/96/2 acetonitrile/water/0.5 mM ammonium formate buffer (pH 3)

Flow Rate: 0.800 mL/min

Gradient:

Time (min)	% A	% B
0.00	0	100
19.00	75	25
19.50	0	100
26.00	0	100

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 4000 Mass Spectrometer

Detector Mode: Negative-ion electrospray

Source Temperature: 500°C

Approximate Retention Time: 20 min

Ions Monitored:

	Transition	Declustering Potential V	Collision Energy eV	Dwell Time ms	Entrance Potential V	Cell Entrance Potential V
Kasugamycinic Acid	379 → 160	-89	-27	100	-5	-7.8
	379 → 178	-69	-30	100	-9	-12

Equipment

Microbalance, Cahn, model number C34/35, serial number C1066/C2251
Centrifuge, Beckman, model number TJ-6, serial numbers 12189, 14659
Microfilterfuge, Biofuge Pico, serial number 40219357
Orbital shaker, model number 3520, serial number 208
Rotovap, model number B-461, serial numbers 1142365, 1141360, 143516
TurboVap, model number N/A, serial number TV0338N11918

Materials

Volumetric flasks, various sizes
Glass syringes, various sizes
Adjustable pipettes, various sizes
500 mL graduated mixing cylinders
250-mL centrifuge bottles
16-mL amber bottles with Teflon-lined caps
10-mL concentrating flasks
0.45 μm centrifuge filters

Chemicals

Acetonitrile, HPLC grade, lot number DD895, Honeywell, Burdick and Jackson
Formic Acid, HPLC grade, lot number SZBA0560, Fluka 99.9% Pure
Methanol, HPLC grade, lot numbers DC312, DC514, Honeywell, Burdick and Jackson
Purified Reagent Water, HPLC grade, lot numbers DC848, DD765-H, DD044-A, DD385-C, Honeywell, Burdick and Jackson
Ammonium Formate, lot number MKBD2004, Aldrich 99.995% Pure
Ammonium Acetate, lot number MKBB7696, Aldrich 99.999% Pure
EDTA, lot number 119K1459, Sigma Aldrich, ACS grade
Sodium Bicarbonate, lot number 45102546, EMD, ACS Grade

Calculations

Calculations were not modified from the original analytical method, except that the concentration in sample extracts was calculated in ng/g instead of ng/mL. Using the calibration curve calculated by linear regression with 1/x weighting, the calculated analyte concentration in the sample extracts in ng/g was calculated using Equation 1:

$$y = mx + b \quad (1)$$

Where:

y	=	Analyte response, peak area
m	=	Slope, calculated by the Analyst® software program
x	=	Calculated analyte concentration in ng/g
b	=	y-intercept, calculated by the Analyst® software program

Equation (1) was rearranged to solve for the analyte concentration (x):

$$x = \frac{(y - b)}{m} \tag{2}$$

The percent recovery of the fortified samples was calculated using Equation 3:

$$\% \text{ Recovery} = \frac{(AC - \text{Average } AC_{UTC})}{FC} \times 100 \tag{3}$$

Where:

AC = Analyte concentration (ng/g)
AC_{UTC} = Analyte concentration in the UTC sample (ng/g)
FC = Concentration fortified (ng/g)

As an example, the LOQ-1 quality control sample, Pyxant ID P2234B03-019 (Figure 13) resulted in a KSMA recovery of 94%. The calculations for this sample are demonstrated below as a representative example of how all the sample results were calculated for this study.

The linear regression analysis of the calibration curve used in the analysis of KSMA residues in soil samples from Trial 2 was determined to have the following regression coefficients: m = 322.24 and b = 1338.6 (Table 5, Figure 2). The analyte peak area (y) was 4360; therefore the concentration of KSMA in the final extract of this sample was calculated using Equation 2:

$$x = \frac{(4360 - 1338.6)}{322.24} = 9.38 \text{ ng/g} \tag{2}$$

The percent recovery of the sample was calculated using Equation 3:

$$\% \text{ Recovery} = \frac{(9.38 \text{ ng/g} - 0.00 \text{ ng/g})}{10.0 \text{ ng/g}} \times 100 = 94\% \tag{3}$$

Statistical Treatment of Data

The mean recoveries for the fortified 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 deviation of the recoveries for a sample was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, %RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Method Modifications

In addition to optimizing the MS parameters, the following method modifications were implemented:

	PTRL West Method 1669W	Pyxant Modifications
Extraction Step 1:	250-mL centrifuge bottles	Amber glass bottles
Extraction Step 2:	0.05 M formic acid in water	0.10 M formic acid in water
Extraction Step 3:	Wrist action shaker	Orbital shaker at 250 rpm
Extraction Step 8:	Microfilterfuge aliquot of extract; dilute if necessary	Microcentrifuged aliquot of extract using 0.45 µ nylon filter for one minute at 2000 rpm; diluted with 0.10 M formic acid in water
Extraction Step 18:	Pear-shaped concentrating flasks	10-mL glass tubes
Extraction Step 19:	• Roto-evaporate to half of the volume (1.25 mL). • Adjust to 1.25 mL with HPLC water.	• Evaporated to near dryness using TurboVap to facilitate blow-down process. • Saturated 20 mM EDTA in water (0.375 mL) was also added in order to inhibit binding of KSMA to iron in the soil extract.
KSM Analysis		
Instrument:	SCIEX API 3000	MDS/Sciex API 4000 to increase signal
Components:	Agilent 1100	Waters Acquity
Column:	Spherex 3µ ODS (C18), 150 × 3.20 mm	SeQuant Zic Hilic, 5 µm, 200A, 4.6 × 15 mm to separate matrix interferences and improve peak shape
KSMA Analysis		
Instrument:	SCIEX API 3000	MDS/Sciex API 4000 to increase signal
Column:	Luna NH2 100A 250 × 4.60 mm	Luna NH2 100A 250 × 4.60 mm; 5 µm particle size was chosen since none specified