I. SUMMARY

Ishihara Sangyo Kaisha (ISK) Biosciences Corporation contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to conduct an Independent Laboratory Validation. The objective of this study was to validate, in surface and drinking (tap) water, the analytical method entitled "Analytical Method for the Determination of SL-160 and its Metabolites DTPU, DTPP, and TPSA in Water by LC-MS/MS" contained in Appendix B of the GPL Final Report "Validation of Method GPL-MTH-082: Analytical Method for the Determination of SL-160 and its Metabolites DTPU, DTPP, and TPSA in Water by LC-MS/MS" (Document Number 140546). This ILV was conducted at the same testing facility as the validation, but was conducted using an independent Study Director and Chemist, separate equipment and instruments, and different lots of reagents. Care was taken to ensure the independence of the ILV staff from those that conducted the original validation. The method was successfully validated using Liquid Chromatography (LC) equipped with a tandem mass spectrometer (MS/MS) detector. The analysis was validated for the determination of SL-160, DTPU, DTPP, and TPSA in surface water during the first method trial. The analysis was validated for the determination of SL-160, DTPU, DTPP, and TPSA in drinking (tap) water during the second method trial. It was determined that the first trial with drinking water was unsuccessful due to SL-160 and DTPP degradation caused by chlorine residues. Before the second trial with drinking water the chlorine was neutralized. The analytical method was validated to demonstrate method ruggedness and to meet US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 Test Guidelines requirements for independent laboratory method validation. The study was conducted under EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160.

Independent Laboratory Validation

One control sample was used for the surface water matrix and two control samples were used for the drinking water matrix in this study. A drinking water sample was obtained from a faucet located at GPL prior to each trial. Prior to the second trial the drinking (tap) water was treated with approximately 5 ppm of sodium bisulfite to neutralize chlorine residues. The surface water sample was obtained from the San Joaquin River at a point near Gravel Haul Road in Fresno, CA. There was no response in the control matrix samples in the chromatograms corresponding to the retention of SL-160, DTPU, DTPP, or TPSA.

Control (untreated) samples of each matrix type were analyzed using the provided analytical method. Samples were diluted with acetonitrile and quantitation was performed using a liquid chromatography mass spectrometry/mass spectrometry system (LC-MS/MS) monitoring two ion transitions for each analyte.

The method was validated at 0.05 and 0.5 µg/L for the detection of SL-160, DTPU, and DTPP in both surface and drinking water. The method was validated at 0.2 and 2 µg/L for the detection of TPSA in both surface and drinking (tap) water. The accuracy and precision data from the successful trials are listed below.

II. MATERIALS

A. Equipment

The equipment that was used is listed below:

- Balance, Analytical, Mettler Toledo XS204
- Volumetric flasks, glass: 10-mL
- Bottles, amber glass with Teflon lined cap: 30, 60, and 120-mL
- Glass vials, clear with Teflon lined caps: 8 and 16-mL
- Volumetric glass pipette: various sizes
- Graduated Cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable micropipettes: 100 µL
- Disposable Pasteur pipettes, glass
- Repeating Pipette, Eppendorf Stream
- HPLC vials, clear glass: 1.8-mL
- AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler

B. Reagents and Standards

The following chemicals were used:

Chemical	Grade	Manufacturer	Distributer	Part No:
Acetonitrile	Optima	Fisher	Fisher	A996-4
Formic Acid (88%)	ACS	Fisher	Fisher	A118P-500
Sodium Bisulfite	ACS	Sigma-Aldrich	Sigma-Aldrich	24397-100G
Water	HPLC	Fisher	Fisher	W5-4

Preparation of Reagent Solutions:

Acetonitrile: HPLC grade water (10:90, v/v): Prepared by adding 100 mL of acetonitrile to 900 mL of HPLC grade water and mixing well.

Mobile Phase A:
0.2% formic acid in acetonitrile: Prepared by adding 2 mL of formic acid to approximately 900 mL of acetonitrile, bringing the volume to 1000 mL with acetonitrile and mixing well.

Mobile Phase B:
0.2% formic acid in HPLC grade water: Prepared by adding 2 mL of formic acid to approximately 900 mL of HPLC grade water, bringing the volume to 1000 mL with additional HPLC grade water and mixing well.

5 mg/mL Sodium Bisulfite Solution: Prepared by weighing approximately 500 mg of Sodium Bisulfite into a 100 mL volumetric flask, bringing the volume to 100 mL with HPLC grade water and mixing well.

1. Reference Substances

The SL-160 analytical reference standard was received in good condition on May 14, 2002. The DTPP and TPSA analytical reference standards were received in good condition on February 13, 2014. The DTPU reference standard was received in good condition on March 26, 2014. All four reference substances were received from Midwest Research Institute, Kansas City, MO. The certificate of analysis for each standard is archived at GPL. The following table contains detailed information for the analytical standards used in this study.

Analytical Standard	CAS #	Lot #	Purity (%)	Expiration Date
SL-160	104040-78-0	Y-920205	100.0	05/03/2018
DTPU	NA	0205	98.5	06/28/2017
DTPP	NA	0205	98.9	06/29/2017
TPSA	104040-76-8	0205	100.0	06/22/2017

Upon receipt, the neat standards were stored in a freezer set to maintain $\leq$ -10 °C (prior to June 18, 2003 the SL-160 reference standard was stored in a freezer set to maintain ≤ 0 °C).

2. Preparation of Standard Solutions

The reference substances were used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

a. Stock Solutions

On March 28, 2014, 10.4 mg of SL-160 reference standard was weighed directly into a 10-mL volumetric flask and diluted to 10 mL with acetonitrile. After correcting for purity, the stock solution contained 1.04 mg/mL SL-160 (Solution A).

On March 28, 2014, 10.6 mg of DTPU reference standard was weighed directly into a 10-mL volumetric flask and diluted to 10 mL with acetonitrile. After correcting for purity, the stock solution contained 1.04 mg/mL DTPU (Solution B).

On March 28, 2014, 10.7 mg of DTPP reference standard was weighed directly into a 10-mL volumetric flask and diluted to

10 mL with acetonitrile. After correcting for purity, the stock solution contained 1.06 mg/mL DTPP (Solution C).

On March 28, 2014, 11.0 mg of TPSA reference standard was weighed directly into a 10-mL volumetric flask and diluted to 10 mL with acetonitrile. After correcting for purity, the stock solution contained 1.10 mg/mL TPSA (Solution D).

b. Intermediate Solutions

Four intermediate solutions were prepared, one for each analyte. A 0.5-mL aliquot of Solution A was diluted to 52 mL with acetonitrile, resulting in a solution that contained 10.0 µg/mL SL-160 (Solution E). A 0.5-mL aliquot of Solution B was also diluted to 52 mL with acetonitrile, resulting in a solution that contained 10.0 µg/mL DTPU (Solution F). A 0.5-mL aliquot of Solution C was diluted to 53 mL with acetonitrile, resulting in a solution that contained 10.0 µg/mL DTPP (Solution G). A 2-mL aliquot of Solution D was diluted to 5.5 mL in acetonitrile, resulting in a solution that contained 400 µg/mL TPSA (Solution H).

A 5-mL aliquot from Solution E, F, and G as well as a 0.5-mL aliquot from Solution H were diluted to 100 mL with acetonitrile, resulting in an intermediate solution that contained 500/500/500/2000 ng/mL SL-160/DTPU/DTPP/TPSA (Solution I).

c. Mixed Fortification Solutions

A 10-mL aliquot of solution I was diluted to 100 mL with acetonitrile, resulting in the high fortification solution containing 50.0/50.0/50.0/200 ng/mL SL-160/DTPU/DTPP/TPSA (Solution J).

Further, a 10-mL aliquot of solution J was diluted to 100 mL with acetonitrile, resulting in the low fortification solution containing 5.00/5.00/5.00/20.0 ng/mL SL-160/DTPU/DTPP/TPSA (Solution K).

A 10-mL aliquot of Solution J was diluted to 100 mL with acetonitrile: HPLC grade water (10:90, v/v), resulting in an intermediate solution containing 5.00/5.00/5.00/20.0 ng/mL SL-160/DTPU/DTPP/TPSA (Solution L). Solution L was used to prepare the calibration standards.

d. Calibration Standards

All calibration standards were diluted into acetonitrile: HPLC grade water (10:90, v/v). The calibration standards were prepared by diluting the solutions as listed in the table as follows:

Initial Solution ID	Volume of Solution (mL)	Final Volume (mL)	Final Solution ID	Standard Concentration (ng/mL)			
				SL-160	DTPU	DTPP	TPSA
L	10	50	M	1.00	1.00	1.00	4.00
L	4	50	N	0.400	0.400	0.400	1.60
L	2	50	O	0.200	0.200	0.200	0.800
L	1	50	P	0.100	0.100	0.100	0.400
L	0.8	100	Q	0.0400	0.0400	0.0400	0.160
L	0.4	100	R	0.0200	0.0200	0.0200	0.0800

C. Safety and Health

Material Safety Data Sheets (MSDS) were available. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. MSDS for the chemicals used in this analysis are located in Appendix D. There were no procedural steps that required special precautions to avoid safety or health hazards.

III. METHODS

A. Principal of Analytical Method

The analysis of surface and drinking (tap) water was performed according to the reference method titled "Analytical Method for the Determination of SL-160 and its Metabolites DTPU, DTPP, and TPSA in Water by LC-MS/MS" (GPL-MTH-082). The limit of quantitation (LOQ) and limit of detection (LOD) were defined for SL-160, DTPU and DTPP as 0.05 µg/L (ppb) and 0.01 µg/L (ppb), respectively. The LOQ and LOD were defined for TPSA as 0.2 µg/L (ppb) and 0.088 µg/L (ppb), respectively.

The first method validation trials for both surface water and drinking water were performed on April 1, 2014. The second method validation trial for drinking water was performed on April 4, 2014. All samples for each validation trial were extracted in one analytical set. Each set consisted of one reagent blank sample (HPLC grade water), two control samples, five LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number; the sample set designation and a sample number (e.g., 547MV01-1).

It was determined that the first trial with drinking water was unsuccessful due to analyte degradation caused by chlorine residues. Before the second trial with drinking water the chlorine was neutralized by addition of approximately 5 ppm of sodium bisulfite to the drinking water matrix. The treated drinking water was then analyzed using the analytical method without change.

Aliquots (10 mL) of control matrix water were fortified and mixed well. An aliquot of each sample (4.5 mL) was added to 0.5 mL acetonitrile and mixed well. Samples were filtered, diluted with acetonitrile: HPLC grade water (10:90) if necessary, vialed and analyzed by LC-MS/MS.

B. Analytical Procedure

1. Control Matrices

a. Drinking Water

The drinking (tap) water control matrix was obtained from the Fresno municipal supply at GPL on 04/01/2014 and on 04/04/2014. The sample collected on 04/04/2014 was treated with a 5 mg/mL solution of sodium bisulfite at the rate of 1 mL sodium bisulfite solution per liter drinking water. Each sample was collected into a 1-L Nalgene bottle and was kept at ambient temperature until its use the day of collection. The characterization of the Fresno municipal supply water is presented in the Water Quality Report located in appendix C.

b. Surface Water

The surface water control matrix was obtained from the San Joaquin River in Fresno, CA at a point near Gravel Haul Road on 03/27/2014. Sub-portions of this sample were labeled with sample number "SJR-032714" and were then transported by GPL personnel to BSK Laboratories in Fresno, California for non-GLP characterization. The non-GLP characterization results of the sample are presented below:

Parameter	Found Value
pH	7.7
Dissolved Oxygen	11 mg/L
Conductivity	60 µmhos/cm
Alkalinity	24 mg/L as $CaCO_3$
Total Hardness	21 mg/L as $CaCO_3$
Total Solids	40 mg/L
Total Organic Carbon	2.2 mg/L
Dissolved Organic Carbon	2.2 mg/L

Sub-portions from the sample "SJR-032714" (refrigerated after collection) were used for the method validation.

2. Preparation of Samples

Sub-samples (10 mL) of the control water matrixes were measured into 16-mL clear glass tubes.

3. Fortifications

Independent laboratory validation samples were fortified at the LOQ or 10x the LOQ. Fortifications were performed using Wiretrol disposable micropipettes to directly fortify the 10-mL samples as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used
LOQ (0.05 µg/L for SL-160, DTPU, and DTPP; 0.2 µg/L for TPSA)	100 µL containing 5.00 ng/mL (SL-160, DTPU, DTPP) or 20.0 ng/mL (TPSA)
10x LOQ (0.5 µg/L for SL-160, DTPU, and DTPP; 2 µg/L for TPSA)	100 µL containing 50.0 ng/mL (SL-160, DTPU, DTPP) or 200 ng/mL (TPSA)

4. Extraction

After fortification and mixing well, an aliquot (4.5 mL) of each sample was added to a vial containing 0.5 mL acetonitrile and mixed well. Samples were filtered through a 0.45 micron PTFE syringe filter, vialed and submitted for analysis by LC-MS/MS.

C. Instrumentation

Instrument:	AB Sciex API4000 LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler
HPLC Column:	Phenomenex Luna C18(2) 50 x 3.00 mm, 3 µm (100 Å) Part # 00B-4251-Y0 Serial # 618924-2
Guard Column:	C18 Security Guard Cartridge 4 x 2.00 mm Part #AJ0-4286
Data System:	Analyst Chromatography Data System version 1.5.2, AB Sciex

Mobile Phases:

A) 0.2% Formic Acid in Acetonitrile
B) 0.2% Formic Acid in Water

Flow Rate: 0.5 mL/minute
Run Time: 8.0 minutes
Injection Volume: 10 µL
Gradient Program:

Time (minutes)	%A	%B
0.0	30	70
5.0	90	10
6.0	90	10
6.1	30	70
8.0	30	70

Column Heater: NA

Approximate Retention Times:

SL-160: 3.2 minutes
DTPU: 2.4 minutes
DTPP: 2.7 minutes
TPSA: 1.5 minutes

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

AB Sciex API-4000 Acquisition Parameters (ESI interface, MRM mode, Positive, Unit/Unit Resolution)						
Analyte	**Ion Pair**	**Q1 (m/z)**	**Q3 (m/z)**	**Dwell (msec)**	**DP**	**CE**
SL-160	Primary	407.9	181.8	50	60	31
	Confirmatory	407.9	139.1	50	60	58
DTPU	Primary	343.9	300.9	50	46	18
	Confirmatory	343.9	281.1	50	46	36
DTPP	Primary	300.9	281.1	50	50	31
	Confirmatory	300.9	238.1	50	50	42
TPSA	Primary	227.0	145.8	50	56	33
	Confirmatory	227.0	126.0	50	56	42

Parameter	Setting
CUR:	25
GS1:	40
GS2:	40
IS:	5500
TEM:	600
CAD:	6
ihe:	ON
EP:	10
CXP:	12

The instrument parameters were optimized for analyte sensitivity and resolution prior to the chromatographic run. The exact parameters were documented with the data set.

D. Potential Interferences

1. Matrix Interference

The detection technique is highly selective for this method. No interferences arising from co-eluting compounds from either water type were observed. Caution should be taken to ensure that any chlorine in water samples is neutralized prior to analysis for SL-160 and DTPP.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

3. Labware Interference

This method uses mostly disposable labware. No interferences from the labware used were observed.

E. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS with monitoring of two ion transition pairs. One ion pair was designated as primary and the second as confirmatory. Confirmatory ion pairs are intended to verify peak identity and may not be useful for quantitation at the LOQ. As this method is highly selective, no additional confirmatory technique was used.

F. Time Required for Analysis

One hour was required for one person to prepare an analysis set from the time samples were prepared to beginning LC-MS/MS analysis. Automated LC-MS/MS analysis was performed overnight. An additional 0.5 hours was spent on data calculation and tabulation the following day. Due to the analysis time (8 minutes per sample), at most, two calendar days are needed to prepare an analysis set and to calculate and tabulate the data.

G. Modification or Potential Problems

There were no modifications to the method. There was one problem encountered. The first trial of the drinking water analysis produced low recoveries for SL-160 and DTPP. Recoveries from the surface water matrix run on the same day produced acceptable recoveries. It was suspected that chlorination in the municipal drinking water system was responsible. A method development analysis showed potential good recovery from tap water aged for 2 days, but low recovery from a fresh tap water sample. This served as evidence to support the role of chlorine disinfectant since it is known to dissipate rapidly in stored water. The Study Director held a phone conversation with the Sponsor on April 3, 2014 to discuss the results and findings. The Sponsor agreed with the assessment that chlorine was the likely cause of low recoveries, and that the tap water should be treated to neutralize chlorine prior to its use in the study. An additional method development experiment was conducted to show that addition of sodium bisulfite to the tap water prior to use allowed acceptable recoveries for all analytes. A second trial was then conducted with tap water treated with 5 ppm sodium bisulfite. The results of the second trial met all acceptance criteria and have been reported.

H. Communication

There was no contact between the team performing the method development and the team conducting the ILV.

Except for the phone call on April 3, 2014 discussing the low recoveries from the tap water trial, there was no contact with the sponsor.

I. Methods of Calculation

Analyst Chromatography Data System version 1.5.2, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations SL-160, DTPU, DTPP, and TPSA as ng/mL using the linear regression function with 1/x weighting. The calibration was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient and other

parameters of the standard curve were calculated. Calibration standards were injected every four to five sample injections as well as at the beginning and end of the injection sequence. Six different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of SL-160, DTPU, DTPP, and TPSA detected in method validation sample extracts were interpolated from the standard calibration curve. The concentration as µg/L of residue found in samples was then calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL \text{ from curve}) \times (\text{Final Vol. in mL}) \times 1\ \mu g \times 1000\ mL}{(\text{Aliquot Volume in mL}) \times 1000\ ng \times 1\ \text{Liter}}$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ \text{Recovery} = \frac{(\text{Measured Concentration, } \mu g/L) \times 100}{(\text{Theoretical Concentration, } \mu g/L\ \text{added})}$$

An example calculation for surface water for a SL-160 laboratory fortification (primary ion) in set 547MV01, sample 547MV01-10 10x LOQ sample fortified at 0.500 µg/L, is as follows:

standard curve equation: $y = 7.49 \times 10^{4}(x) + 108$

where x = *SL-160 concentration in ng/mL and*

y = *peak response* = 3539.6

SL-160 concentration from the curve =0.0458 *ng/mL*

$$\mu g/L = \frac{(0.0458\ ng/mL\ SL\text{-}160) \times (50\ mL) \times 1\mu g \times 1000\ mL}{(4.5\ mL) \times 1000\ ng \times 1\ \text{Liter}} = 0.509\ \mu g/L$$

$$\%\ \text{recovery} = \frac{0.509\ \mu g/L}{0.500\ \mu g/L} \times 100 = 102\%$$

No detectable residues were measured in any control samples. Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

J. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), coefficient of variation and 95% confidence interval for analyte recovery data. Linear regression analysis was applied to LC-MS/MS calibration curves for the determination of slope, y-intercept and correlation coefficient values.