1. INTRODUCTION

The objective of this study was to independently validate the Golden Pacific Laboratories residue method GPL-MTH-077 for the determination of IKF-1216 (fluazinam) and five metabolites (AMPA, DAPA, CAPA, DCPA, and HYPA) in water. The independent validation (ILV) was conducted using untreated control samples of tap water. Tap water is considered representative for the intent of the method.

The method was found to be suitable for the determination of fluazinam and five metabolites in water over the concentration range 0.1 ng/mL to 1 ng/mL with a validated limit of quantitation (LOQ) of 0.1 ng/mL.

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. 8.1 (2, 3, 4). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 850.7100 (5), OPPTS 835.6200 (6) and PR Notice 96-1 (7). This validation report presents the results of the independent laboratory validation for fluazinam and five metabolites in water.

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 tap water samples. The independent laboratory has no organizational ties to Golden Pacific Laboratories, and used all of its own equipment and supplies, so that there was no common link between the Sponsor or Golden Pacific Laboratories and the Study Director or analyst. Throughout the conduct of the study, any communications between the Sponsor or Golden Pacific Laboratories and the Study Director and/or the analyst were logged for inclusion in the report. No one from the Sponsor or Golden Pacific Laboratories 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.

2. ANALYTICAL

2.1 Sample Receipt, Labeling and Storage

This control matrix was acquired from the independent laboratory. The control sample was assigned unique master logbook (MLB) number 99997553 and stored frozen (approximately -20°C).

2.2 Preparation of Solutions and Standards

The analytical reference standard/test substance utilized during the independent laboratory method validation is summarized below. The reference standards were received from the Sponsor, assigned unique MLB numbers, and stored frozen, protected from light. The Certificates of Analysis are included in Appendix B.

Standard	MLB Number	Percent Purity	Recertification Date	Lot Number
Fluazinam (IKF-1216)	00007302	99.98%	April 12, 2012	Y010920
AMPA	00007304	99.02%	April 4, 2012	9511
DAPA	00007305	99.54%	April 9, 2012	9209
CAPA	00007502	99.5%	April 20, 2016	9012
DCPA	00007503	99.9%	April 21, 2016	8604-5
HYPA	00007303	99.8%	April 21, 2016	0205

The following stock solutions were prepared in acetone to obtain nominal concentrations of 1.00 mg/mL and stored refrigerated (2-8°C) when not in use:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [mg/mL]*
Fluazinam	Standard	N734P01-1	51.4	50.0	1.03
AMPA	Standard	N734P01-2	25.7	25.0	1.02
DAPA	Standard	N734P02-1	25.6	25.0	1.02

The following stock solutions were prepared in acetonitrile to obtain nominal concentrations of 1.0 mg/mL and stored frozen (≤ -20°C) when not in use:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [mg/mL]*
CAPA	Standard	N734P8-1	26.0	25.0	1.03
DCPA	Standard	N734P8-2	27.1	25.0	1.08
HYPA	Standard	N734P9-1	26.0	25.0	1.04

*Resulting concentrations after correcting for purity

Mixed intermediate and fortification solutions were prepared in acetonitrile and stored frozen (≤ -20°C) when not in use:

From Solution Lot Number	Concentration [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N734P01-1	1030	97.1	100	1.00	N734P24-1 and N734P27-1
N734P01-2	1020	98.0			
N734P02-1	1020	98.0			
N734P8-1	1030	97.1			
N734P8-2	1080	92.6			
N734P9-1	1040	96.2			
N734P24-1	1.00	5000	50	0.100	N734P24-2
N734P27-1	1.00	5000	50	0.100	N734P27-2
N734P8-2	1080	92.6	100	1.00	N734P28-1
N734P28-1	1.00	5000	50	0.100	N734P28-2
N734P28-2	0.100	5000	50	0.0100	N734P29-1

Mixed calibration standards were prepared in 10/90 acetonitrile/water (v/v) with 0.02% formic acid and stored frozen (≤ -20°C) when not in use:

From Solution Lot Number	Concentration [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N734P01-1	1030	97.1	100	1.00	N734P27-1
N734P01-2	1020	98.0			
N734P02-1	1020	98.0			
N734P8-1	1030	97.1			
N734P9-1	1040	96.2			
N734P24-2	0.100	5000	50	0.0100	N734P25-1

Concentration of Stock Solution [ng/mL]	Aliquot of Stock Solution [µL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]	Final Solution Lot Number
10.0	5000	25	2.00	N734P26-1
10.0	1000	10	1.00	N734P26-2
10.0	5000	100	0.500	N734P26-3
2.00	1000	10	0.200	N734P26-4
1.00	1000	10	0.100	N734P26-5
0.500	1000	10	0.0500	N734P26-6

2.3 Fortification of Recovery Samples

The ILV trial of the method was performed for fluazinam and five metabolites in tap water. The trial was comprised of one batch, 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 with fluazinam and five metabolites at 0.1 ng/mL, the LOQ of the method

5 (five) control samples fortified with fluazinam and five metabolites at 1 ng/mL, 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 [ng/mL]	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [mg/L]
100 mL	0.1	0.100	0.100
	1.0	0.100	1.00

For DCPA only:

Specimen Portion	Nominal Target Fortification Level [ng/mL]	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [mg/L]
100 mL	0.1	1.00	0.0100
	1.0	1.00	0.100

2.4 Sample Analysis

The ILV trial was conducted as described in the Golden Pacific Laboratories residue analytical method GPL-MTH-077 (1) with minor modifications (see Section 3.4).

The sample extracts for the analysis of fluazinam and five metabolites were analyzed directly by HPLC employing mass spectrometric detection (LC/MS/MS).

For more specific details, refer to the analytical method (1).

2.5 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. Confirmatory ion transitions were monitored. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

2.5.1 Instrumentation

Typical HPLC Conditions

Instrument:	Applied Biosystems API 5000 LC/MS/MS System (System 15) equipped with Shimadzu 10AD Autosampler for glass vials and pumps
Column:	Synergy Polar-RP, 4 µm, 2.0 × 50 mm
Temperature:	Ambient
Injection Volume:	20-50 µL
Run Time:	7.5 minutes
Mobile Phase:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Total Flow Rate:	500 µL/min
Gradient:	

Time, min	% A	% B
0.00	90	10
4.00	20	80
5.00	20	80
6.50	90	10
7.50	90	10

Typical MS Conditions

Mass Spectrometer:	Applied Biosystems API 5000 Mass Spectrometer
Detector Mode:	Positive-ion electrospray
Source Temperature:	550°C
Ions Monitored:	

	Transition	Declustering Potential V	Collision Energy eV	Dwell Time ms	Retention Time (+/- 0.3 min.)	
					Quant. Ion	Conf. Ion
Fluazinam	465.2 → 373.0 465.2 → 338.0	110	35	100	5.40	4.80
AMPA	435.1 → 373.1 435.1 → 354.0	110	35	100	5.10	4.50
DAPA	404.8 → 333.1 404.8 → 353.0	110	35	100	4.90	4.40
CAPA	441.2 → 349.0 441.2 → 303.0	110	35	100	4.80	4.30
DCPA	417.2 → 325.1 417.2 → 278.9	110	30	100	3.90	4.40
HYPA	447.0 → 355.0 447.0 → 383.1	110	29	100	4.80	4.80

2.5.2 Equipment

Analytical Balance, Sartorius, model number AC 120S, serial number 20103137 (EQ37)

2.5.3 Materials

Volumetric flasks, various sizes
Adjustable pipettes, various sizes
250-mL amber glass vials
1.8 mL clear glass HPLC vials

2.5.4 Chemicals

Acetone, HPLC grade, lot number 50337, Honeywell
Acetonitrile, HPLC grade, lot number DE080, DE188, Honeywell
Formic acid, HPLC grade, lot numbers SZBA320V, Fluka
Purified reagent water, HPLC Grade, lot number DD947-D, Honeywell
Sterile water, AVD67913, Thermo

2.6 Calculations

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

$$y = mx + b \tag{1}$$

Where:

x = is the analyte concentration in ng/mL
y = analyte peak area
m = Slope, calculated by the Analyst ® software program
b = y-intercept, calculated by the Analyst ® software program

Equation 1 was rearranged as Equation 2 to solve for the analyte concentration.

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

The analyte concentration found in final extract (ng/mL) is given by Equation 3:

$$AC = x \times DF \tag{3}$$

Where:

AC = Analyte concentration found in final extract (ng/mL)
DF = Dilution factor (total dilution volume ÷ extract aliquot volume)

The percent recovery of the fortified samples was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{\text{AC}}{\text{FC}} \times 100 \qquad (4)$$

Where:
AC = Analyte concentration in ng/mL
FC = Concentration fortified (ng/mL)

As an example, the 10×LOQ quality control sample, Pyxant ID P2326B02-010 (Figure 27) resulted in a fluazinam recovery of 92%. 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 fluazinam residues in water samples from Trial 2 was determined to have the following regression coefficients: $m = 2.08E+05$ and $b = -277$ (Figure 1). The analyte peak area (y) was 1.71E+05; therefore the concentration of fluazinam in the final extract of this sample was calculated using Equation 2:

$$x = \frac{(1.71E+05+277)}{2.08E+05} = 0.823 \text{ ng/mL} \qquad (2)$$

The final concentration of fluazinam found in the sample in ng/mL was calculated using Equation 3:

$$\text{AC} = 0.823 \text{ ng/mL} \times 1.12 = 0.922 \text{ ng/mL} \qquad (3)$$

The percent recovery of the sample was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{0.922 \text{ ng/mL}}{1.00 \text{ ng/mL}} \times 100 = 92.2\% \qquad (4)$$

2.7 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.

3.4 Method Modifications

Section 7.0 of the method states that a 9-mL aliquot of a 100-mL fortified sample is to be taken and combined with a 1-mL aliquot of 0.2% formic acid in acetonitrile. In order to reduce sample transfer steps but maintain the same ratio, 11 mL of 0.2% formic acid in acetonitrile were added to each 100-mL sample.

Minor instrument parameters such as injection volume and gradient were adjusted to optimize sensitivity.

3.5 Critical Steps

With one exception, there were no steps encountered with the methodology that were found to need to be followed so exactly that they were considered critical to the success of the method for the determination of fluazinam and five metabolites in water.

Section 4.0 of the method states "Unless otherwise indicated, the equipment listed below may be substituted with functionally equivalent equipment." However, there is no indication that the clear glass HPLC vials may not be substituted.

Additionally, Section 7.0 of the method states that plastic bottles should not be used "because more than one analyte of interest may adhere to the plastic," then specifies the potential recovery losses of fluazinam and AMPA.

The LC/MS/MS system used in this study (System 15) is equipped with a Shimadzu SIL-20AC-HT autosampler, which is not capable of handling the glass vials specified in the method. For this reason, a polypropylene 96-well block was used in Trial 1 instead.

After unacceptable correlation coefficients (r) and incongruous recovery results were obtained for all analytes, it was determined that due to analyte adherence, the polypropylene 96-well plates were not suitable for sample analysis.

New solutions and samples were prepared for Trial 2. A Shimadzu 10AD autosampler was used with System 15 instead, which is capable of handling the glass vials. Trial 2 results were acceptable for all analytes.

Based on the unacceptable results obtained in Trial 1, it should be emphasized in Section 4.0 and Section 7.0 that no substitutions should be made for glass equipment and that all analytes may adhere to the plastic.