Method Validation Study for the Determination of Residues of Propyzamide and its Metabolites in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry

INTRODUCTION

Scope

This method is applicable for the quantitative determination of residues of propyzamide and its RH-24644, RH-24655, RH-24580, RH-26059, and UK1 metabolites in water (surface water, ground water and drinking water). The method was validated over a concentration range extending from the limit of quantitation at 0.05 µg/L to an upper range which encompassed the level of quantitation of 1.0 µg/L for each analyte. The common name, chemical name, molecular formula, and structure for each of these compounds are given in Table 1.

The validation study was conducted to comply with the requirements of the U.S. EPA Ecological Effects Test Guidelines, OPPTS 850.7100 (1); EU Council Directive 91/414/EEC with particular regard to Section 4 of SANCO/3029/99 rev. 4 and Section 2,6 of SANCO/825/00 rev. 8; and the PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (2-4).

Method Principle

An aliquot of 4.0 mL of water is mixed with 1.0 mL of acetonitrile. The sample is then centrifuged and approximately 1 mL of the solution is transferred to an autosampler vial. For each sample set, two injections are performed for the LC/MS/MS analysis in both the positive and negative electrospray ionization (ESI) mode.

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents, and procedures used in this method before commencing laboratory work. Sources of information include: operation manuals, material safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance with applicable governmental requirements.

Acetonitrile and acetone is flammable and should be used in well-ventilated areas away from ignition sources. Formic acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Test Substance/Analytical Standard and Internal Standard

The following test substances were obtained from the Test Substance Coordinator, Dow AgroSciences LLC. The common name, chemical name, molecular formula, and structure for each of these compounds are given in Table 1.

Analytical Standard	TSN Number	Percent Purity	Recertification Date	Reference
RH-23315 (Propyzamide)	TSN105825	98.2%	23-Jun-2013	FAPC09-227036
RH-24644	TSN029409-0001	99.0%	1-Sep-2012	FAPC10-267038
RH-24580	TSN103029	99.3%	1-Sep-2014	FAPC10-267039
RH-24655	TSN103034	95.41%	30-Sep-2013	FAPC11-000123
RH-26059	TSN103038	99%	1-Sep-2013	FAPC11-000122
UK1	TSN103018	99%	1-Sep-2014	FAPC10-261115

Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware are used to prepare analytical standards, fortification solutions, and calibration standards.

Laboratory Equipment

Balance, analytical, Model AE100, Mettler-Toledo, Inc.

Centrifuge, Allegra™ 6, Beckman Coulter.

Pipet, positive-displacement, 10-100 µL capacity, catalog number MR-100, Gilson Inc.

Pipet, positive-displacement, 250 µL capacity, catalog number M250, Gilson Inc.

Pipet, positive-displacement, 100-1000 µL capacity, catalog number MR-1000, Gilson Inc.

Pipetter, repeater, Eppendorf, order number 4981 000.019, Eppendorf North America.

Pipetter, adjustable, Eppendorf, 50-1000 µL, catalog number 21-378-83, Brinkmann Instruments.

Vortex mixer, Model G-560, Scientific Industries, Inc.

Chromatographic System

Column, analytical, Zorbax SB-C8, 4.6 mm x 75 mm, 3.5 µm particle size, catalog number 866953-906, Agilent Technologies.

Liquid chromatograph, Symbiosis Pharma, Spark Holland Inc.

Mass spectrometer, Model API 5000, MDS/Sciex.

Mass spectrometer data system, Model Analyst 1.5.1, MDS/Sciex.

Glassware and Materials

Bottle, 1.0 L, media bottle, catalog number 06-423-3D, Fisher Scientific.

Bottle, 2.0 L, media bottle, catalog number 06-423-3D, Fisher Scientific.

Cylinder, graduated, 250 mL, catalog number 08-570-F, Fisher Scientific.

Cylinder, graduated, 1000 mL, catalog number 08-570-H, Fisher Scientific.

Cylinder, graduated, 2000 mL, catalog number 08-566-11H, Fisher Scientific.

Flask, volumetric, 20 mL, catalog number 10-209F, Fisher Scientific.

Flask, volumetric, 10 mL, catalog number 10-209F, Fisher Scientific.

Flask, volumetric, 5 mL, catalog number 10-209F, Fisher Scientific.

Pipet tip, positive-displacement, 100 µL capacity, catalog number CP100, Gilson Inc.

Pipet tip, positive-displacement, 250 µL capacity, catalog number CP250, Gilson Inc.

Pipet tip, positive-displacement, 1000 µL capacity, catalog number CP1000, Gilson Inc.

Combitips plus, 10 mL, order number 0030 069.269, Eppendorf North America.

Tubes, culture, 16x100 mm, with screw cap, catalog number 99449-16, Corning Products.

Tubes, disposable, culture, 16x100 mm, catalog number 14-961-29, Fisher Scientific

Vial, 2 Dram, 8 mL, screw thread, with PTEF cap, ART No. 60940A 8, Kimble Chase

Vial, 3 Dram, 12 mL, screw thread, with PTEF cap, ART No. 60940A 12, Kimble Chase

Scintillation vial, disposable, catalog number 03-337-26, Fisher Scientific.

HPLC autosampler vials, catalog number C4000-1W, National Scientific.

Reagents

Acetonitrile, CHROMASOLV for HPLC ≥99.9%, catalog number 439134-4L, Sigma-Aldrich.

Formic acid, Optima, LC/MS grade, catalog number A117-50, Fisher Scientific.

Water, CHROMASOLV for HPLC ≥99.9%, catalog number 270733-4L, Sigma Aldrich.

Acetone, CHROMASOLV for HPLC ≥99.9%, catalog number 270725-4L, Sigma Aldrich.

Prepared Solutions

Acetonitrile with 0.1% formic acid (mobile phase A)

Measure 2000 mL of acetonitrile using a graduated cylinder, and transfer the solvent into a 2 L media bottle. Remove 2 mL of acetonitrile from the bottle with pipette. Add 2 mL formic acid to the bottle with pipette. Cap the bottle and mix well.

Water with 0.1% formic acid (mobile phase B)

Measure 2000 mL of water using a graduated cylinder, and transfer the solvent into a 2 L media bottle. Remove 2 mL of acetonitrile from the bottle with pipette. Add 2 mL formic acid to the bottle with pipette. Cap the bottle and mix well.

water/acetonitrile (80/20)
Measure 800 mL of water using a graduated cylinder, and transfer the solvent into a 1 L media bottle. Measure 200 mL of acetonitrile using a graduated cylinder, and transfer the solvent into the same 1 L media bottle. Cap the bottle and mix well.

EXPERIMENTAL

Instrumental Conditions

Typical LC/MS/MS Conditions

Positive acquisition mode for RH-23315, RH-24644, RH-24655, RH-24580, and UK1,

Instrumentation:	Symbiosis Pharma AB SCIEX API 5000 LC/MS/MS System AB SCIEX Analyst 1.5.1 data system
Column:	Zorbax SB-C8, 3.5 µm, 75x4.6 mm
Column Temperature:	Ambient
Injection Volume:	30 µL
Injection Wash Program	1) 2 x 700 µL acetone 2) 2 x 700 µL acetonitrile 3) 2 x 700 µL water
Run Time:	approximately 8.0 minutes
Mobile Phase:	A – acetonitrile with 0.1% formic acid B – water with 0.1% formic acid
Flow Rate:	0.8 mL/min (~300 µL/min split to source)

Gradient

Time (min)	A %	B %
0:01	10	90
3:00	100	0
5:00	100	0
5:15	10	90
7:00	10	90

Flow Diverter Program: 1) 0.0→2.0 min: flow to waste
2) 2.0→5.0 min: flow to the source
3) 5.0→end of the run: flow to waste

Interface: ESI
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – unit, Q3 – unit
Curtain Gas (CUR): 15
Collision Gas (CAD): 4.0
Temperature (TEM): 500 °C
Ion Source Gas 1 (GS1): 50
Ion Source Gas 2 (GS2): 50

Period 1

Time: 5.0 minutes
Acquisition Delay 1.0 minutes
Smart Settling: Off
Settling Time: 0 ms
MR Pause: 5 ms

Transitions:	Q1 Ions (m/z)	Q3 Ions (m/z)	Time (ms)	DP/CE/CXP
RH-23315 (256/190)	256.000	190.000	50	46/21/12
RH-23315 (258/192)	258.000	192.000	50	46/21/12
RH-24644 (256/173)	256.000	172.900	50	80/33/10
RH-24644 (256/109)	256.000	109.000	50	80/73/10
RH-24655 (258/190)	258.100	190.000	50	60/17/12
RH-24655 (258/173)	258.100	173.000	50	60/31/18
RH-24580 (274/173)	274.000	173.000	50	58/31/24
RH-24580 (274/109)	274.000	109.000	50	58/75/16
UK1 (222/156)	222.100	156.000	50	50/19/22
UK1 (222/139)	222.100	139.000	50	50/29/20

Negative acquisition mode for RH-26059

Instrumentation:	Symbiosis Pharma AB SCIEX API 5000 LC/MS/MS System AB SCIEX Analyst 1.5.1 data system
Column:	Zorbax SB-C8, 3.5 µm, 75x4.6 mm
Column Temperature:	Ambient
Injection Volume:	30 µL
Injection Wash Program	1) 2 x 700 µL acetone 2) 2 x 700 µL acetonitrile 3) 2 x 700 µL water
Run Time:	approximately 8.0 minutes
Mobile Phase:	A – acetonitrile with 0.1% formic acid B – water with 0.1% formic acid
Flow Rate:	0.8 mL/min (~300 µL/min split to source)

Gradient

Time (min)	A %	B %
0:00	10	90
3:00	100	0
5:00	100	0
5:15	10	90
7:00	10	90

Flow Diverter Program:	1) 0.0→2.0 min: flow to waste 2) 2.0→5.0 min: flow to the source 3) 5.0→end of the run: flow to waste
Interface:	ESI
Polarity:	Negative
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	15
Collision Gas (CAD):	4.0
Temperature (TEM):	500 °C
Ion Source Gas 1 (GS1):	50
Ion Source Gas 2 (GS2):	50

Period 1

Time:	5.0 minutes
Acquisition Delay	1.0 minutes
Smart Settling:	Off
Settling Time:	0 ms
MR Pause:	5 ms

Transitions:	Q1 Ions (m/z)	Q3 Ions (m/z)	Time (ms)	DP/CE/CXP
RH-26059 (288/188)	288.169	188.000	50	-90/-22/-17
RH-26059 (290/190)	290.169	190.000	50	-90/-22/-17

Full-scan and product-ion mass spectra for propyzamide and its RH-24644, RH-24655, RH-24580, RH-26059, and UK1 metabolites are shown in Figures 1-6, respectively.

Typical calibration curves for the quantitative determination of propyzamide and its RH-24644, RH-24655, RH-24580, RH-26059, and UK1 metabolites in water are shown in Figures 7-18.

Typical chromatograms of standards, control samples, a 0.05 µg/L (0.05 ng/mL, LOQ) recovery samples, and 1.0 µg/L (1.0 ng/mL) recovery samples for the quantitative determination of propyzamide and its RH-24644, RH-24655, RH-24580, RH-26059, and UK1 metabolites in water are illustrated in Figures 19-28, respectively.

Preparation of Stock Solutions and Spiking Solutions

Weigh out approximately 20 mg of propyzamide (RH-23315), RH-24644, RH-24655, RH-24580, RH-26059, and UK1 and quantitatively transfer each analyte into separate 20 mL volumetric flasks with acetonitrile. Dilute to volume to obtain stock solutions for each of the analytes. Exact concentration of each stock solution is calculated and recorded (approximately 1 mg/mL).

Transfer appropriate amount of above stock solutions into the same 20 mL volumetric flask using a positive displacement pipette. Dilute to volume with acetonitrile to obtain a 10 µg/mL mixed spiking solution.

Prepare additional mixed spiking solutions by further diluting the above 10 µg/mL mixed spiking solution with acetonitrile as follows:

Parent Spiking Solution Conc.[a]	Volume of Parent Spiking Solution	Volume of Final Spiking Solution	Final Spiking Solution Conc.	Equivalent Sample Conc.[b]
µg/mL	µL	mL	ng/mL	µg/L (ng/mL)
10.0	100	10.0	100	1.0
(0.10)	500	10.0	5.0	0.05
(0.10)	150	10.0	1.5	0.015

[a] Numbers in parenthesis indicate the solution for series dilution.
[b] The equivalent samples concentration is based on fortifying a 4.0 mL water sample with 40 µL of spiking solution.

Sample Origin, Numbering, Preparation and Storage

Untreated control samples were obtained from the Dow AgroSciences LLC Sample Management Group. All samples were tracked in the Dow AgroSciences LLC Regulatory Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation is included in the study file.

Sample Group Number	Water Type	pH	Hardness (mg equiv. $CaCO_3$/L)	Total Suspended Solids (ppm)	Alkalinity (mg $CaCO_3$/L)	Total Organic Carbon (ppm)	Dissolved Organic Carbon (ppm)
001	(Monitoring Well) Ground Water	8.2	330	6	183	3.4	2.2
002	(Pond) Surface Water	8.0	135	8	80	8.0	6.9
003	(Tap) Drinking Water	8.6	4	8	292	3.1	2.6

No sample preparation was required for the water samples prior to analysis. Samples were stored refrigerated at approximately 4 °C after their time of sampling and during the course of the method validation study, except when they were removed for taking aliquots for sample analysis.

Analysis Procedure

1. In 2 dram (8 mL) vials, freshly prepare calibration standards with spiking solutions and water/acetonitrile (80/20) on the day of analysis as follows:

Spiking Solution Conc.[a]	Volume of Spiking Solution	Volume of Solvent	Final Volume of Calibration Std.	Final Calibration Std. Conc.	Equivalent Sample Conc.[b]
ng/mL	µL	µL	µL	ng/mL	µg/L
100	80	4920	5000	1.6	2.0
(1.6)	1000	333	1333	1.2	1.5
(1.6)	1000	1000	2000	0.8	1.0
(0.8)	1000	1000	2000	0.4	0.5
5.0	80	2420	2500	0.16	0.2
(0.16)	1000	1000	2000	0.08	0.1
1.5	80	2920	3000	0.04	0.05
(0.04)	1000	2333	3333	0.012	0.015

[a] Numbers in parenthesis indicate solutions for series dilution.
[b] The equivalent sample concentration is based on a diluting factor of 1.25.

2. Pipet approximately 1.0 mL of the above calibration standard into an autosampler vial for LC/MS/MS analysis.

3. For reagent blanks, transfer 4.0 mL of HPLC water into 16x100 mm disposable culture tube with PTFE-lined screw-top cap. Add 40 µL of acetonitrile, then 1.0 mL of acetonitrile. Vortex for a few seconds to mix well.

4. For control samples, transfer 4.0 mL of water samples (surface water, ground water and drinking water) into the disposable culture tube. Add 40 µL of acetonitrile, then 1.0 mL of acetonitrile. Vortex for a few seconds to mix well.

5. For fortified samples, transfer 4.0 mL of control water sample into disposable culture tube. Add 40 µL of the appropriate spiking solution to obtain fortified samples at LOD, LOQ and a higher concentration level (0.015, 0.05 and 1 µg/L, respectively). Add 1.0 mL of acetonitrile to each sample. Vortex for a few seconds to mix well.

6. Centrifuge the samples for 10 minutes at 2000 rpm.

7. Pipet approximately 1.0 mL of the sample into an autosampler vial for LC/MS/MS analysis.

8. Two injections are performed for samples and calibration standards, with one injection in the positive acquisition mode for RH-23315, RH-24644, RH-24655, RH-24580 and UK1, and the other injection in the negative acquisition mode for RH-26059.

Calculations

Inject the series of calibration standards described in the calibration standard preparation section using the conditions listed in the Instrument Conditions Section and determine the peak areas for the analytes.

Prepare a standard curve using power regression analysis with no weighting by plotting the analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) as shown in Figures 7-18.

Determine the concentration (ng/mL) and recovery (%) for the sample as described in the example calculation outlined in Figure 29 for propyzamide from sample set 110587 S01.

Confirmation of Residue Identity

The method is highly selective for the determination of propyzamide and its metabolites in water by virtue of the chromatographic separation and the selective detection system. When detection is by tandem mass spectrometry, confirmation of the presence of the analyte should require the observation of a precursor ion plus a structurally significant product ion observed at the same retention time (*5*). To demonstrate further confirmation, a total of two MRM ion transitions are monitored for each analyte.

RH-23315 (Propyzamide)	Q1/Q3 *m/z* 256/190 (quantitation) Q1/Q3 *m/z* 258/192 (confirmation)
RH-24644	Q1/Q3 *m/z* 256/173 (quantitation) Q1/Q3 *m/z* 258/109 (confirmation)
RH-24655	Q1/Q3 *m/z* 258/190 (quantitation) Q1/Q3 *m/z* 258/173 (confirmation)
RH-24580	Q1/Q3 *m/z* 274/173 (quantitation) Q1/Q3 *m/z* 274/109 (confirmation)
RH-26059	Q1/Q3 *m/z* 288/188 (quantitation) Q1/Q3 *m/z* 290/190 (confirmation)
UK1	Q1/Q3 *m/z* 222/156 (quantitation) Q1/Q3 *m/z* 222/139 (confirmation)

Inject the series of calibration standards described in the Analysis Procedure Section using the conditions listed in the Instrumental Conditions Section and determine the peak areas for the analyte as indicated below. The example calculation is given for the analyte propyzamide (RH-23315).

For each standard, calculate the respective confirmation ratios.

$$\text{Confirmation Ratio} = \frac{\text{RH - 23315 confimatory peak area}}{\text{RH - 23315 quantitative peak area}}$$

$$\text{Confirmation Ratio} = \frac{\text{RH - 23315 peak area at } m/z \text{ 258/192}}{\text{RH - 23315 peak area at } m/z \text{ 256/190}}$$

For example, using the data for RH-23315 from the 0.04 ng/mL standard of set 110587 S01:

$$\text{Confirmation Ratio} = 5850/8490 = 0.689$$

A percent difference for each recovery sample can be calculated by comparing the confirmation ratio of the recovery sample with the average confirmation ratio of the standards. The percent difference for each recovery sample should fall within the range of ±20% of the average confirmation ratio of the standards.

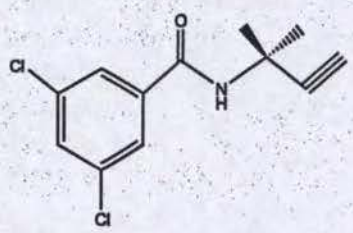
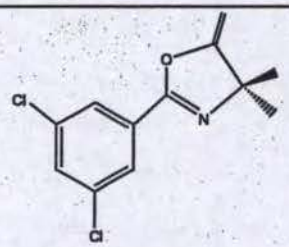
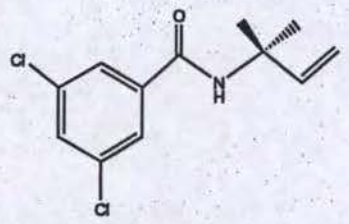
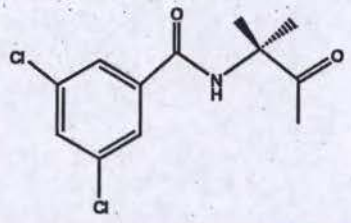
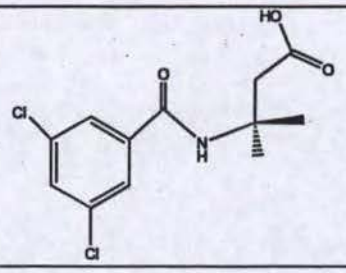
Statistical Treatment of Data

Statistical treatment of data included the calculation of regression equations, correlation coefficients (r) for describing the linearity of calibration curves, means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

Assay Time

A typical analytical run would consist of eight calibration standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of three fortified controls (one of which must be at the LOQ), and 34 samples. This typical analytical run requires approximately 3 hours for sample preparation, followed by the LC/MS/MS analysis.

Table 1. Identities and Structures of Propyzamide and Its Metabolites

Identifying information	Chemical Structure
Propyzamide (Pronamide, RH-23315) IUPAC name: 3,5-dichloro-N-(1,1-dimethylprop-2-ynyl)benzamide Chemical Formula: $C_{12}H_{11}Cl_2NO$ Molecular Weight: 256.13 Nominal Mass: 255 CAS Number: 23950-58-5	
RH-24644 IUPAC name: 3,5-dichloro-N-(1,1,2-trimethylprop-2-enyl)benzamide Chemical Formula: $C_{12}H_{11}Cl_2NO$ Molecular Weight: 256.13 Nominal Mass: 255 CAS Number: 29918-40-9	
RH-24655 IUPAC name: 3,5-dichloro-N-(1,1 dimethylpropenyl) benzamide Chemical Formula: $C_{12}H_{13}Cl_2NO$ Molecular Weight: 258.14 Nominal Mass: 257 CAS Number: NA	
RH-24580 IUPAC name: 3,5-dichloro-N-(1,1-dimethyl-2-oxopropyl)benzamide Chemical Formula: $C_{12}H_{13}Cl_2NO_2$ Molecular Weight: 274.14 Nominal Mass: 273 CAS Number: 29918-41-0	
RH-26059 IUPAC name: 3-[(3,5-dichlorobenzoyl)amino]-3-methylbutanoic acid Chemical Formula: $C_{12}H_{13}Cl_2NO_3$ Molecular Weight: 290.14 Nominal Mass: 289 CAS Number: NA	

RH-23801 (UK1, 3-chloro kerb) IUPAC name: 3-chloro-N-(1,1-dimethylpropynyl)benzamide Chemical Formula: $C_{12}H_{12}ClNO$ Molecular Weight: 221.68 Nominal Mass: 221 CAS Number: NA