Abstract/Summary

This report summarizes a successful independent laboratory validation (ILV) of the analytical method for the analysis of valifenalate and its metabolites valifenalate acid and p-chlorobenzoic acid in water.

Objective

The objective was to independently validate an analytical method [1] for the determination of valifenalate, valifenalate acid and p-chlorobenzoic acid (PCBA) in water to achieve a limit of quantitation (LOQ) at 0.1 µg/L for each analyte.

Method Principles

Valifenalate, valifenalate acid and PCBA in water were extracted by solid phase extraction (SPE). Appropriate aliquot of an acidified portion of the water sample containing the three analytes was allowed to pass through a conditioned Oasis HLB cartridge. Methanol was used as an eluent to elute out the analytes from the SPE tube. The eluate was diluted (1:1 v/v) with Milli-Q water for LC/MS/MS analysis. Valifenalate and valifenalate acid were analyzed on the LC/MS/MS in positive ionization mode while PCBA was analyzed under negative ionization mode and all three analytes achieved an LOQ of 0.1 µ/L.

1. Introduction

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of Valifenalate and its two metabolites: Valifenalate Acid and p-Chlorobenzoic acid (PCBA) in water. The analysis was performed by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) based on the method described in "Ferguson, L, 2015: Method Validation - Determination of Residues of Valifenalate and its Metabolites Valifenalate Acid and p-Chlorobenzoic Acid in Water. Ricerca Document No. 032663-1, FMC Tracking No. 2014RES-VAL1464" [1]. The ILV was conducted on surface water matrix targeting an LOQ of 0.1 µg/L for the analytes.

2. Experimental

2.1 Test System

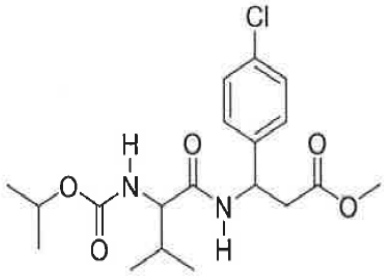
The validation study was carried out on surface water collected from Jacob's Pond - 42°09'33.9"N 70°50'51.5"W. The surface water was characterized by Agvise (Appendix 2)**.**

2.2 Analytical Test and Reference Substances

Reference standards of valifenalate, valifenalate acid and PCBA were provided by the Sponsor (Appendix 1).

Valifenalate (IR5885):

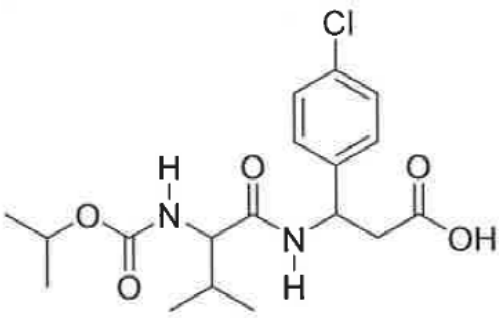
Structure:



Empirical formula:	$C_{19}H_{27}ClN_2O_5$
Molecular weight:	398.88 g/mol
CAS No.:	283159-90-0
Batch No.:	20071/77
Expiry Date:	November 2017
Purity:	99.27%

Valifenalate acid (IR5839):

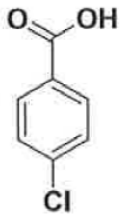
Structure



Empirical formula:	$C_{18}H_{25}ClN_2O_5$
Molecular weight:	384.86 g/mol
CAS No.:	283159-90-0
Batch No.:	G029/08
Expiry Date:	21 February 2017
Purity:	98.4%

p-Chlorobenzoic acid (PCBA)

Structure:



Empirical Formula:	$ClC_6H_4CO_2H$
Molecular Weight:	156.57 g/mol
CAS No.:	74-11-3
Batch No:	LC07337V
Expiry Date:	May 2017
Purity:	99.2%

2.3 Analytical Method

The Analytical Method Report, 'Ferguson, L, 2015: Method Validation - Determination of Residues of Valifenalate and its Metabolites Valifenalate acid and p-Chlorobenzoic acid in Water' from Ricerca Biosciences [1] was used to conduct the ILV.

2.3.1 Apparatus

2.3.1.1 Laboratory Equipment

- Balances:
  - Ohaus Explorer EOD120, SN D2771118362156
  - Mettler AT201, SN L92660
- Centrifuges:
  - Sorvall Legend XF
  - Eppendorf 5810
- Turbovap – Zymark TurboVap II
- Pipettors: Rainin Pipet-plus, various sizes
- Fisherbrand 50 mL polypropylene centrifuge tubes
- Fisher glass vials, 25 mL screw-cap and 2 mL crimp top
- Fisher 15 mL PP centrifuge tubes
- Gas tight syringes – Hamilton, various sizes

All reusable glassware were cleaned in a laboratory dishwasher, solvent rinsed, and air-dried before use. Consumable glassware (injection vials, glass pipettes) were baked at 400°C for at least 30 min before use.

2.3.1.2 LC-MS/MS System

- Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler
- Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source
- Thermo Betasil C18 100 x 2.1 mm, 5 µm Catalog # 70105102130 for analysis of valifenalate and valifenalate acid
- ARMOR C18, 5 µm, 100 x 2.1 mm, P/N ADV7009 for analysis of PCBA

2.3.2 Solvents, Chemicals and Consumables

- Methanol, HPLC grade, Fisher, lot #152153
- Formic Acid, Acros Organics, lot #B0527746
- Acetone, Fisher, lot # 150933
- Hydrochloric acid 0.5N, Fisher lot # 153033

2.3.3 Preparation of Standard Solutions

2.3.3.1 Stock and Working Solutions

Valifenalate, valifenalate acid and PCBA were received as neat compounds from the Sponsor. The neat material were stored under ambient conditions when not in use. The stock solutions were prepared in methanol and stored in the freezer at -20 C.

Stock solutions:

Compound	Battelle ID	Neat material ID	Mass (mg)	Final Volume (mL)	Solution Concentration (µg/mL)**
Valifenalate	IM13	150624-04 (Val*)	10.78	10	1070
Valifenalate Acid	IN93	150624-05 (ValA*)	10.74	10	1057
p-Chlorobenzoic Acid	IO10	150624-03 (PCBA*)	10.19	10	1011
Valifenalate	IN38	150624-04 (Val)	10.11	10	1004
Valifenalate Acid	IN98	150624-05 (ValA)	10.45	10	1028
p-Chlorobenzoic Acid	IO11	150624-03 (PCBA)	9.05	10	898

* “Val” refers to valifenalate, “ValA” refers to valifenalate acid, and PCBA refers to p-chlorobenzoic acid.
** Concentration corrected for purity of neat material

Working solutions: The working solutions were prepared in methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Working solution: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IN94	IM13	1070	467	10	50.0 (val)
	IN93	1057	473		50.0 (valA)
IN95	IN94	50.0	1000	10	5.00 (val)
		50.0			5.00 (valA)

Working solution: PCBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO12	IO10	1011	990	10	100
IO13	IO12	100	1000	10	10.0

2.3.3.2 Fortification Solutions

Fortification solutions of the analytes were prepared in methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Fortification solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IN96	IN95	5.00	1000	10	0.500 (val)
		5.00			0.500 (valA)
IN97	IN96	0.500	1000	10	0.0500 (val)
		0.500			0.0500 (valA)

Fortification solutions: PCBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO14	IO13	10.0	1000	10	1.00
IO63	IO14	1.00	1000	10	0.100

2.3.3.3 Solvent Calibration Solutions:

Intermediate solutions for calibrations were prepared in methanol:water (50:50 v:v) with 0.1 % formic acid and stored in the freezer at -20 °C when not in use:

Intermediate calibration solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO01	IN38	1004	500	10	50.2 (val)
	IN98	1028	485		49.9 (valA)
IO02	IO01	50.2	500	10	2.51 (val)
		49.9			2.49 (valA)
IO03	IO02	2.51	500	10	0.125 (val)
		2.49			0.125 (valA)
IP40	IN38	1004	500	10	50.2 (val)
	IN98	1028	485		49.9 (valA)
IP41	IP40	50.2	500	10	2.51 (val)
		49.9			2.49 (valA)
IP42	IP41	2.51	500	10	0.125 (val)
		2.49			0.125 (valA)

Intermediate calibration solutions: PCBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (µg/mL)
IO15	IO11	898	110	10	9.88
IO16	IO15	9.88	100	10	0.0988
IO17	IO16	0.0988	1000	10	0.00988

Solvent calibration solutions were prepared by diluting working calibration standards in methanol:water (20:80 v:v) with 0.1% formic acid. Solvent calibration solutions were stored in a freezer at -20 °C when not in use:

Calibration solutions: valifenalate and valifenalate acid					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (ng/mL)
IO04	IO03	0.125	20	10	0.251 (val)
		0.125			0.249 (valA)
IO05	IO03	0.125	40	10	0.502 (val)
		0.125			0.499 (valA)
IO06	IO03	0.125	120	10	1.51 (val)
		0.125			1.50 (valA)
IO07	IO03	0.125	400	10	5.02 (val)
		0.125			4.99 (valA)
IO08	IO03	0.125	800	10	10.0 (val)
		0.125			9.97 (valA)
IO09	IO02	2.51	60	10	15.1 (val)
		2.49			15.0 (valA)
IP43	IP42	0.125	20	10	0.251 (val)
		0.125			0.249 (valA)
IP44	IP42	0.125	40	10	0.502 (val)
		0.125			0.499 (valA)
IP45	IP42	0.125	120	10	1.51 (val)
		0.125			1.50 (valA)
IP46	IP42	0.125	400	10	5.02 (val)
		0.125			4.99 (valA)
IP47	IP42	0.125	800	10	10.0 (val)
		0.125			9.97 (valA)
IP48	IP42	0.125	1200	10	15.1 (val)
		0.125			15.0 (valA)

Calibration solutions: PCBA					
Battelle ID	Use solution	Stock conc. (µg/mL)	Stock volume (µL)	Total volume (mL)	Conc. (ng/mL)
IO69	IO66	0.0988	75	10	0.741
IO70	IO66	0.0988	150	10	1.48
IO71	IO66	0.0988	300	10	2.96
IO72	IO66	0.0988	500	10	4.94
IO73	IO66	0.0988	1000	10	9.88
IO74	IO66	0.0988	1500	10	14.8

Matrix matched calibration solutions of the analytes were prepared by diluting 500 µL of untreated control (UTC) extract with a combination of water and standard solution. Matrix-matched calibration solutions were stored refrigerated with samples at 0 - 4 °C when not in use:

Battelle ID	Stock solution ID	Volume taken of stock solution (µL)	Volume of water added	Final Volume (µL)	Solution Conc. (ng/mL)
CG761UTC-AG(5)	IO08	25	425	1000	0.251 (val)
					0.249 (valA)
	IO74	50			0.741 (PCBA)
CG761UTC-AG(7)	IO08	50	350	1000	0.502 (val)
					0.499 (valA)
	IO74	100			1.481 (PCBA)
CG761UTC-AG(9)	IO08	150	150	1000	1.51 (val)
					1.50 (valA)
	IO74	200			2.96 (PCBA)
CG761UTC-AG(11)	IO03	40	410	1000	5.02 (val)
					4.99 (valA)
	IO66	50			4.94 (PCBA)
CG761UTC-AG(13)	IO03	80	320	1000	10.0 (val)
					10.0 (valA)
	IO66	100			9.88 (PCBA)
CG761UTC-AG(15)	IO03	120	230	1000	15.1 (val)
					15.0 (valA)
	IO66	150			14.8 (PCBA)

2.3.4 Extraction

Extraction Method

1. Using graduated cylinder, 100 mL aliquots of surface water samples were added into Erlenmeyer flasks and fortified, if necessary
2. Six mL [500 mg] Oasis HLB cartridges were conditioned with 5 mL methanol followed by 5 mL of Milli-Q water
3. One mL of 1 N HCl was added to the sample aliquot in the Erlenmeyer flask
4. Reservoirs and adapters were attached to the Oasis HLB cartridges and transferred the acidified water sample to the cartridge
5. The samples were allowed to flow through at 40 drops per minute with the aided by a light vacuum.
6. Twenty mL of water and 1 mL of 1 N HCl were added to the Erlenmeyer flask to rinse the flask and transfer the rinsates to the cartridge
7. The water sample and the acidified rinsates were allowed to pass through the cartridge
8. The cartridge was eluted with 5 mL of methanol into a 15 mL centrifuge tube.
9. Five mL of Milli-Q water was added to the centrifuge tube and vortexed to mix the contents
10. An aliquot was transferred to an HPLC auto-sampler vial
11. Analysis was performed by LC-MS/MS

2.4 LC-MS/MS Analysis

Calibration solutions, matrix-matched calibration solutions, blank extracts, control sample extracts and fortified sample extracts were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The following LC/MS/MS conditions were used for valifenalate and valifenalate acid analysis in positive ionization mode:

<table>
<tr><td>LC System</td><td colspan="4">Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler</td></tr>
<tr><td>LC Column</td><td colspan="4">Thermo Betasil C18 100 x 2.1 mm, 5 µm Catalog # 70105102130</td></tr>
<tr><td>Injection Vol.</td><td colspan="4">10 µL</td></tr>
<tr><td rowspan="10">HPLC Method</td><td colspan="4">Mobile Phase A: 0.1 % formic acid in water</td></tr>
<tr><td colspan="4">Mobile Phase B: 0.1 % formic acid in acetonitrile</td></tr>
<tr><td colspan="4">Mobile Phase Composition</td></tr>
<tr><td>Time (min)</td><td>Flow rate (mL/min)</td><td>% A</td><td>% B</td></tr>
<tr><td>0.0</td><td>0.8</td><td>80</td><td>20</td></tr>
<tr><td>1.5</td><td>0.8</td><td>80</td><td>20</td></tr>
<tr><td>1.7</td><td>0.8</td><td>5</td><td>95</td></tr>
<tr><td>3.0</td><td>0.8</td><td>5</td><td>95</td></tr>
<tr><td>3.1</td><td>0.8</td><td>80</td><td>20</td></tr>
<tr><td>5.0</td><td>0.8</td><td>80</td><td>20</td></tr>
<tr><td>Ret. Times</td><td colspan="4">~ 2.2 – 2.4 minutes</td></tr>
<tr><td>MS/MS System</td><td colspan="4">Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source</td></tr>
<tr><td rowspan="8">Ion Source Conditions ESI Positive Polarity</td><td colspan="2">Source temperature:</td><td colspan="2">550°C</td></tr>
<tr><td colspan="2">Gas supply (GS 1):</td><td colspan="2">70 (arbitrary units)</td></tr>
<tr><td colspan="2">Gas supply (GS 21):</td><td colspan="2">70 (arbitrary units)</td></tr>
<tr><td colspan="2">Curtain gas (CUR):</td><td colspan="2">45 (arbitrary units)</td></tr>
<tr><td colspan="2">Collision gas (CAD):</td><td colspan="2">medium (arbitrary units)</td></tr>
<tr><td colspan="2">Entrance potential:</td><td colspan="2">10 V</td></tr>
<tr><td colspan="2">IonSpray voltage:</td><td colspan="2">4000 V</td></tr>
<tr><td colspan="2">Resolution:</td><td colspan="2">Q1: Unit, Q3: Unit</td></tr>
</table>

The following LC/MS/MS conditions were used for PCBA analysis in negative ionization mode:

<table>
<tr><td>LC System</td><td colspan="4">Shimadzu LC2080 UHPLC system, including a vacuum solvent degasser, binary UHPLC pump, column oven, autosampler</td></tr>
<tr><td>LC Column</td><td colspan="4">ARMOR C18, 5 µm, 100 x 2.1 mm, P/N ADV7009</td></tr>
<tr><td>Column Temp</td><td colspan="4">40 °C</td></tr>
<tr><td>Injection Vol.</td><td colspan="4">20 µL</td></tr>
<tr><td rowspan="8">HPLC Method</td><td colspan="4">Mobile Phase A: 0.1 % formic acid in water</td></tr>
<tr><td colspan="4">Mobile Phase B: 0.1 % formic acid in acetonitrile</td></tr>
<tr><td colspan="4">Mobile Phase Composition</td></tr>
<tr><td>Time (min)</td><td>Flow rate (mL/min)</td><td>% A</td><td>% B</td></tr>
<tr><td>0.0</td><td>0.8</td><td>90</td><td>10</td></tr>
<tr><td>2.5</td><td>0.8</td><td>10</td><td>90</td></tr>
<tr><td>3</td><td>0.8</td><td>10</td><td>90</td></tr>
<tr><td>3.1</td><td>0.8</td><td>90</td><td>10</td></tr>
<tr><td>5</td><td>0.8</td><td>90</td><td>10</td></tr>
<tr><td>Ret. Times</td><td colspan="4">~ 2.08 – 2.14 minutes</td></tr>
<tr><td>MS/MS System</td><td colspan="4">Applied Biosystems MDS Sciex API 6500 linear ion trap MS/MS system with TurboIonspray (ESI) source</td></tr>
<tr><td rowspan="8">Ion Source Conditions ESI Positive Polarity</td><td colspan="2">Source temperature:</td><td colspan="2">550°C</td></tr>
<tr><td colspan="2">Gas supply (GS 1):</td><td colspan="2">50 (arbitrary units)</td></tr>
<tr><td colspan="2">Gas supply (GS 21):</td><td colspan="2">50 (arbitrary units)</td></tr>
<tr><td colspan="2">Curtain gas (CUR):</td><td colspan="2">20 (arbitrary units)</td></tr>
<tr><td colspan="2">Collision gas (CAD):</td><td colspan="2">medium (arbitrary units)</td></tr>
<tr><td colspan="2">Entrance potential:</td><td colspan="2">-10 V</td></tr>
<tr><td colspan="2">IonSpray voltage:</td><td colspan="2">-4500 V</td></tr>
<tr><td colspan="2">Resolution:</td><td colspan="2">Q1: Unit, Q3: Unit</td></tr>
</table>

MRM Transitions for Valifenalate, Valifenalate acid and PCBA

MS/MS Conditions for Valifenalate	399.5 m/z > 155.0 m/z (used for quantitation)			
	Dwell time:	100 msec	DP:	80 V
	CE:	39 V	CXP:	11 V
	399.5 > 116.0 m/z (used for confirmation)			
	Dwell time:	100 msec	DP:	80 V
	CE:	25 V	CXP:	10 V
MS/MS Conditions for Valifenalate Acid	385.3 > 116.0 m/z (used for quantitation)			
	Dwell time:	100 msec	DP:	85 V
	CE:	27 V	CXP:	10 V
	385.3 > 144.0 m/z (used for confirmation)			
	Dwell time:	100 msec	DP:	85 V
	CE:	19 V	CXP:	9 V
MS/MS Conditions for PCBA	155.0 m/z > 111.0 m/z (used for quantitation)			
	Dwell time:	500 msec	DP:	-27
	CE:	-16 V	CXP:	-10 V
	155.0 > 35.0 m/z (used for confirmation)			
	Dwell time:	500 msec	DP:	-19 V
	CE:	-45 V	CXP:	-16 V

2.5 Calculations

The following equation was used to calculate the individual residues R in mg/kg:

$$R = C_{End} \times \left(\frac{V_{Exl}}{W}\right)$$

Where:

R: Residue in µg/L.
C_{End}: Final concentration of analyte in extract in ng/mL.
V_{Exl}: Total volume of the eluate (10 mL).
W: Total volume of the sample (100 mL)

The values reported in the tables are calculated with full precision, but displayed with three significant figures.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$$Rec. = \frac{R}{R_{fort.}} \times 100$$

Where

Rec.: Recovery (%)
$R_{fort.}$: Residue fortified, in µg/L.

The calculation is exemplified with the water sample CG762LOQ-AG(0)fortified at 0.100 µg/L (LOQ) for valifenalate. The final extract was examined by LC-MS/MS run to give a peak area of 48108 counts for the transition 399.5 m/z > 155.0 m/z. Using the respective calibration curve (see Figure 7) a final concentration of 0.950 ng/mL was calculated (see Table 2).

Thus:

$$R = C_{End} \times \left(\frac{V_{El}}{W}\right) = 0.950\,\frac{\text{ng}}{\text{mL}} \times \left(\frac{10\text{ mL}}{100\ mL}\right) = 0.0950\,\frac{\mu\text{g}}{\text{L}}$$

And:

$$Rec. = \frac{R}{R_{fort.}} \times 100 = \frac{0.0950\,\frac{\mu\text{g}}{\text{L}}}{0.100\,\frac{\mu\text{g}}{\text{L}}} \times 100 = 95.0\%$$