Bull. Environ. Contam. Toxicol. (1994) 52:538–545

Environmental Contamination and Toxicology

Quantification of 2,4-D and Related Chlorophenoxy Herbicides by a Magnetic Particle-Based ELISA

T. S. Lawruk,[1] C. S. Hottenstein,[1] J. R. Fleeker,[2] J. C. Hall,[3] D. P. Herzog,[1] F. M. Rubio[1]

[1]Ohmicron Corporation, 375 Pheasant Run, Newtown, Pennsylvania 18940, USA
[2]North Dakota State University, Biochemistry Department, P.O. Box 5516, Fargo, North Dakota 58105, USA
[3]University of Guelph, Department of Environmental Biology, Ontario, N1G 2W1, Canada

Received: 27 May 1993/Accepted: 2 August 1993

Pesticide testing of water, food and soil has increased dramatically over the past several years due to concerns over the potential contamination of drinking, surface, and groundwater from spills, spraying and run-off. Current testing methods for the chlorophenoxy herbicides are time-consuming and expensive, and require specialized instrumentation and often analyte derivatization for quantitation. Immunological assays provide the analytical chemist with a sensitive, rapid, and reliable method suitable for both lab and field analysis, and allow cost-effective sample evaluation without solvent disposal or analyte derivatization (Van Emon and Lopez-Avila 1992).

2,4-Dichlorophenoxyacetic acid (2,4-D) is a selective systemic herbicide commonly used for the post-emergence control of annual and perennial broad-leaved weeds in cereals, maize, sorghum, grasslands, established turf, sugar cane, rice, forests, and non-crop land. Treatment of areas adjacent to water allows the control of broad-leaved aquatic weeds. 2,4-D salts are readily absorbed by the roots, and the esters are readily absorbed by the foliage. Esters of 2,4-D are rapidly hydolyzed to 2,4-D in the environment. 2,4-D is classified by the EPA as a category III contaminant with a maximum contaminant level of 70 ppb in drinking water and a Practical Quantitation Level of 5 ppb (USEPA 1991).

The principles of enzyme-linked immunosorbent assay (ELISA) have been described (Hammock and Mumma 1980) and applied to the detection of 2,4-D in water (Fleeker 1987; Hall et al. 1989). These ELISAs used polystyrene wells or tubes on which antibody or hapten-protein conjugate were passively adsorbed. The desorption of antibody or other proteins which have been passively adsorbed to a solid-phase are major factors that adversely affect assay sensitivity and precision. Magnetic particle-based ELISAs have previously been described and applied to the detection of pesticide residues (Rubio et al. 1991; Itak et al. 1992, 1993; Lawruk et al. 1992, 1993;). These ELISAs eliminate the imprecision problems associated with coated plates and tubes (Harrison et al. 1989) by the precise addition of the antibody which is covalently coupled to a magnetic solid-phase. The uniform dispersion of particles throughout the reaction mixture provides superior reaction kinetics (Newman and Price 1991). In the present work, we describe the development and evaluation of a competitive ELISA for the quantification of 2,4-D and its esters in environmental water samples using magnetic particles as the solid-phase for the 2,4-D antibody.

Correspondence to: T. S. Lawruk

MATERIALS AND METHODS

Amine-terminated superparamagnetic particles of approximately 1-μm diameter were obtained from Advanced Magnetics, Inc. (Cambridge, MA). Superparamagnetic particles separate quickly in weak magnetic fields but have no magnetic memory which allows for repeated magnetic separations and resuspension of the particles. The small particle size permits the particles to stay suspended in solution for over one hour. The following special reagents were also used: glutaraldehyde (Sigma Chemical, St. Louis, MO), rabbit anti-2,4-D serum (J.C. Hall, University of Guelph, Ontario, Canada), 2,4-D-HRP conjugate (Ohmicron, Newtown, PA), hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Labs, Gaithersburg, MD), humic acid (Fluka Chemical, Buchs, Switzerland), 2,4-D USEPA Certified Reference Standard, 5000 ppm in acetonitrile, 99.9% pure (NSI Environmental Solutions, Research Triangle Park, NC), and 2,4-D and related compounds as well as non-related crossreactants (Riedel-de-Haen, Hanover, Germany). All other reagents were reagent grade or of suitable high purity.

The following apparatus were used: Adjustable pipette, Gilson P-1000 (Rainin, Woburn, MA), repeating pipette (Eppendorf, Hamburg, Germany), RPA-I Analyzer™ (Ohmicron), and magnetic separation rack (Ohmicron).

The rabbit anti-2,4-D coupled magnetic particles were prepared by glutaraldehyde activation of the magnetic solid-phase (Rubio et al. 1991).

All water samples were assayed by adding 250 μL of sample, 250 μL of 2,4-D-HRP conjugate, and 500 μL of anti-2,4-D magnetic particles to a polystyrene test tube. After incubating for 30 minutes at room temperature, the magnetic rack was used to magnetically separate the reaction mixture (Itak et al. 1992). The magnetic particles were washed twice with 1.0 mL of deionized water to remove unbound conjugate and eliminate potential interfering substances. The colored product was developed for 20 minutes at room temperature by the addition of 500 μL of hydrogen peroxide/TMB solution. The color reaction was stopped and stabilized with 500 μL of 2M sulfuric acid. The absorbance at 450 nm and 2,4-D concentration for each sample were determined using the RPA-I Analyzer. Since the enzyme-labeled 2,4-D competes with the unlabeled (sample) 2,4-D for antibody binding sites, the color developed is inversely proportional to the concentration of 2,4-D in the sample. The observed sample results were compared to a linear regression line using a natural logarithm (ln) of the herbicide concentration versus B/Bo standard curve (where B/Bo is the absorbance at 450 nm observed for a sample or standard divided by the absorbance at the zero standard). The calibrators were prepared in the zero standard (0.025M Tris/ 0.15M NaCl/ 0.1% BSA preserved solution) and contained 2,4-D at 0, 1.0, 10.0, and 50.0 ppb .

RESULTS AND DISCUSSION

Figure 1 illustrates the mean standard curve for the 2,4-D calibrators collected over 55 runs, error bars represent ±2 SD. The inhibition of the enzyme labeled 2,4-D from binding the 2,4-D antibody coupled magnetic particle at the 1.0 ppb level is significant (87% B/Bo). The assay sensitivity based on 90% B/Bo (Midgley et al. 1969) is 0.7 ppb. This sensitivity is comparable with the method detection limit reported for EPA Method 515 of 1.2 ppb (USEPA 1986), the drinking water Maximum Contaminant Level (MCL) of 70 ppb, and the Practical Quantitation

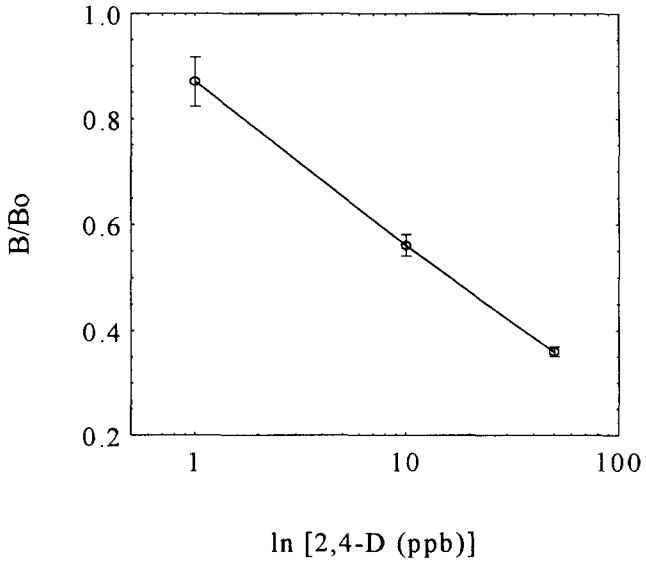

Figure 1. Dose response curve for 2,4-D. Each point represents the mean of 55 determinations. Vertical bars indicate ± 2 SD about the mean.

Table 1. Precision of 2,4-D measurement in the ELISA.

Sample	1	2	3
Replicates	5	5	5
Days	5	5	5
N	25	25	25
Mean ppb	4.16	19.6	36.1
% CV Intra-assay	9.5	8.7	7.7
% CV Inter-assay	11.5	11.1	11.3

Level (PQL) of 5 ppb (USEPA 1991). Fleeker (1987) has previously reported two ELISAs with limits of quantitation of 1.0 and 5.0 ppb. An indirect ELISA developed by Hall et al. (1989) has a working range of 100 to 10,000 ppb.

A precision study in which unfiltered surface and groundwater samples were spiked with 2,4-D at 5, 20, and 40 ppb, and each assayed five times per assay on five different days is shown in Table 1. The within and between day variation was determined by analysis of variance (ANOVA) (Bookbinder and Panosian 1986). Coefficients of variation (%CV) within and between day were less than 10% and 12%, respectively.

Table 2 summarizes the accuracy of the 2,4-D ELISA analyzed by adding known amounts of 2,4-D to four water samples obtained locally, including water from a small creek, bay, well, and municipal source. The accuracy was assessed by assaying the samples before and after the addition of 2,4-D, and correcting for the initial 2,4-D concentration. Added amounts of 2,4-D were recovered correctly in all cases with an average assay recovery of 103%. In addition, an EPA-certified 2,4-D

Table 2. Accuracy of 2,4-D ELISA

2,4-D added (ppb)	Mean (ppb)	*n*	SD (ppb)	% Recovery
5.00	4.6	8	0.4	92
15.0	16.8	8	1.5	112
30.0	33.0	8	3.3	110
40.0	39.4	8	3.2	99
Average				103

Table 3. Recovery of a reference standard in the 2,4-D ELISA

2,4-D Added (ppb)	2,4-D Recovered (ppb)	% Recovery
2.0	1.9	95
5.0	4.5	90
10.0	10.0	100
15.0	15.2	101
20.0	22.4	112
25.0	24.5	98
30.0	29.3	98
40.0	36.8	92
Average		98

reference standard was added to deionized water at various concentrations and the amount recovered was determined (Table 3). The excellent recovery in both cases suggests that no sample matrix problems or interferences were present in the samples tested, and the accuracy of the ELISA is linear across the range of the assay.

A well validated ELISA must demonstrate "parallel" dilution of positive samples compared to the standards used in the assay. If positive results were due to either

Table 4. Linearity upon sample dilution in the 2,4-D ELISA

Sample ID	Undiluted	1:2	1:4	1:8
Sample 1				
assayed (ppb)	41.8	22.4	10.8	4.5
expected (ppb)	41.8	20.9	10.5	5.2
recovery (%)	-	107	103	86
Sample 2				
assayed (ppb)	41.9	23.6	10.8	5.2
expected (ppb)	41.9	20.9	10.5	5.2
recovery (%)	-	113	103	100
Sample 3				
assayed (ppb)	30.4	17.2	8.0	4.2
expected (ppb)	30.4	15.2	7.6	3.8
recovery (%)	-	113	105	110

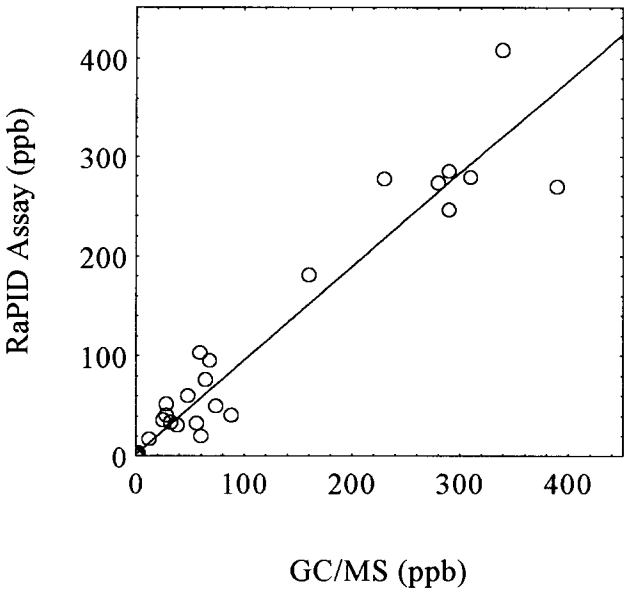

Figure 2. Correlation between 2,4-D concentrations as determined by ELISA and GC/MS methods. $n = 56$, $r = 0.970$, $y = 0.938x + 2.21$.

specific or non-specific interferences, the values of the diluted samples would not assay as expected (i.e., the difference between expected and observed values would increase with increasing dilution). Values obtained from three spiked surface water samples diluted in the zero standard showed agreement between measured and expected values (Table 4). The expected values were calculated from the 2,4-D concentration of the undiluted sample.

Correlation of 56 groundwater samples analyzed by this ELISA method (y) and an established gas chromatography/mass spectroscopy method (x) is illustrated in Figure 2. The regression analysis yields a correlation (r) of 0.970 and a slope of 0.938 between methods. For the GC/MS method, 200 mL of methylene chloride was shaken with one liter of acidified (pH less than 2.0) water sample and allowed to separate. The organic layer was concentrated to one milliliter and diazomethane was added to methylate the 2,4-D. One microliter of the 2,4-D methyl ester solution produced was injected into the GC/MS for analysis.

The 50% inhibition concentration (I_{50}) was determined by estimating the amount of chlorophenoxy analogue necessary to displace 50% of the 2,4-D-HRP. The least detectable dose (LDD) for each compound was determined as the concentration of analog required to achieve 90% B/Bo, the limit of detection of the ELISA. The percent crossreactivity was calculated as the I_{50} of the analog divided by the I_{50} of 2,4-D (times 100). Table 5 summarizes the specificity data using a variety of chlorophenoxy analogs and many structurally unrelated agricultural compounds. The ELISA is up to twenty times more sensitive to the ester analogs of 2,4-D.

Various components commonly found in water, such as inorganic salts and humic acid were added to water samples. These water samples were evaluated by the

Table 5. Specificity of the 2,4-D ELISA

Compound	LDD (ppb)	I_{50} (ppb)	% Cross-reactivity
2,4-D	0.70	15.0	100
2,4-D propylene glycol ester	0.05	0.79	1960
2,4-D ethyl ester	0.05	0.82	1890
2,4-D isopropyl ester	0.07	1.44	1080
2,4-D methyl ester	0.12	1.64	945
2,4-D butyl ester	0.19	2.40	646
2,4-D sec-butyl ester	0.13	2.20	705
2,4-D butoxyethyl ester	0.13	3.10	500
2,4,5-T methyl ester	0.98	18.1	106
2,4-D butoxypropylene ester	1.21	31.1	50
2,4-D isooctyl ester	2.08	30.0	52
2,4,5-T	2.98	190	8.2
2,4-DB	3.95	139	11.2
MCPA	7.8	159	10.0
Silvex methyl ester	12.4	1000	1.9
MCPB	56.8	1470	1.1
4-Chlorophenoxyacetic acid	61.1	1220	1.3
Dichlorprop	117	7500	0.2
2,4,5-TP (Silvex)	167	2060	0.8
Dichlorophenol	217	3570	0.4
Triclopyr	830	NR	<0.1
MCPP	1160	NR	<0.1

NR = No reactivity up to 10,000 ppb
LDD = Least Detectable Dose (at 90% B/Bo)
The following pesticides were assayed at 10,000 ppb and found to have no reactivity in the assay: alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide, atrazine, benomyl, butylate, captan, captafol, carbaryl, carbendazim, carbofuran, dicamba, 1,3-dichloropropene, dinoseb, metolachlor, metribuzin, pentachlorophenol, picloram, simazine, terbufos, thiophanat-methyl and thiabendazol.

Table 6. Effect of possible interfering substances in the 2,4-D ELISA

Compound	Max. Conc. Tested (ppm)	0 ppb Sample	30.0 ppb Sample
Nitrate	250	ND	27.6
Copper	250	ND	31.4
Nickel	100	ND	25.7
Thiosulfate	250	ND	26.2
Sulfite	250	ND	26.0
Sulfate	10,000	ND	27.9
Iron	250	ND	29.3
Magnesium	250	ND	31.2
Calcium	250	ND	26.1
NaCl	0.65M	ND	32.0
Humic acid	50	ND	30.3
Silicates	1,000	ND	28.2

ND = none detected <0.7 ppb

ELISA, before and after the addition of 30 ppb 2,4-D. Table 6 summarizes that no interference occurred up to the tested levels. The concentration chosen for each compound were those that would exceed levels found in most groundwater samples (American Public Health Association 1989). In addition, the assay was not affected by changes in pH in the range from 3 to 11 (data not shown), therefore, environmental water samples with a wide range of pH can be assayed with this ELISA without a sample interference or neutralization.

This work describes a magnetic particle-based ELISA for the detection of 2,4-D and related chlorophenoxy herbicides, and its performance characteristics using water samples. All the principles of sound quality control used for traditional chromatographic methods should be applied to ELISA methods. Understanding the unique aspects of immunochemical techniques described, will enhance the user's ability to produce reliable results. The ELISA allows for the screening of large numbers of samples, to identify those that require more extensive analysis. The assay compares favorably to GC/MS determinations and eliminates the need for sample derivatization and solvent disposal. The ELISA exhibits excellent precision and accuracy which can provide consistent monitoring of environmental samples. The magnetic particle-based system is rapid and more sensitive than previously reported ELISAs (Fleeker 1987; Hall et al. 1989) and EPA method 515 (USEPA 1986) for 2,4-D residue detection. Also, the assay sensitivity of 0.7 ppb (90% B/Bo) in water, exceeds the EPA Maximum Contaminant Level in drinking water of 70 ppb. The antibody employed allows for the detection of 2,4-D and related chlorophenoxy compounds in the presence of other pesticides and commonly found groundwater components. This ELISA provides results in less than one hour without the problems of coating variability and antibody desorption, encountered with coated tubes and microtiter plates, (Howell et al. 1981; Engvall 1980; Lehtonen and Viljanen 1980) and is ideally suited for the adaptation to on-site monitoring of 2,4-D in water, soil, food and solid waste.

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