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Immunoassays for the Detection of 2,4-D and Picloram in River Water and Urine

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Immunoassays for 2,4-D [(2,4-dichlorophenoxy)acetic acid] and picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) detection were developed with polyclonal antibodies raised in New Zealand white rabbits. Concentrations of 2,4-D within the working range 100–10 000 and 50–10 000 ng/mL could be quantitated with an indirect enzyme-linked immunosorbent assay (ELISA) and a radioimmunoassay (RIA) in river water and urine, respectively. Concentrations of picloram within the working range 50–5000 ng/mL also could be quantitated in river water and urine by RIA. Determinations using the immunoassays required no sample cleanup. Specificities of the antisera for structurally similar herbicides were low compared to 2,4-D or picloram. The RIA methods incorporated a novel radiolabel consisting of [^{3}H]glycine covalently linked to the herbicide molecule. When compared to the ELISA, the RIA was a more simple, efficient, and rapid procedure, requiring fewer steps to complete the assay. The immunoassays would be suitable for herbicide quantitation in applicator exposure and environmental fate studies.

The potential of immunochemical technology for pesticide analysis has been examined by Hammock and Mumma (1980) and more recently by Van Emon et al. (1985) and by Cheung et al. (1988). Immunoassays are proposed for pesticides that are difficult to analyze by standard techniques. Many pesticides, including 2,4-D and picloram, require an extensive sample preparation including derivatization before they can be analyzed by gas chromatography. As alternative methods, immunoassays can be sensitive, specific, and precise, providing for rapid, cost-effective analyses.

Current concerns about potential health hazards connected with pesticide use have focused on 2,4-D as a suspected cancer-causing agent (Hoar et al., 1986). As a broadleaf weed killer, 2,4-D is used extensively in field crops, on turf, and in noncrop lands. Its widespread use and associated health concerns have made monitoring environmental and biological samples for the presence of 2,4-D desirable. Among the types of samples monitored are well waters for 2,4-D contamination (Frank et al., 1987) and urine samples for applicator exposure studies (Grover et al., 1986; Libich et al., 1984).

Picloram is used for the control of woody and broadleaf herbaceous plants. It is relatively resistant to breakdown in the environment and has been found to be mobile in the soil (Hamaker et al., 1963). Picloram residues have been found in surface and groundwater samples (Frank et al., 1987; Baur et al., 1972). The mobility in the environment shown by picloram along with the susceptibility of certain crops to extremely small amounts of this compound (Ragab, 1975) makes monitoring water for picloram residues necessary.

Radioimmunoassays (RIA) for 2,4-D (Rinder and Fleeker, 1981; Knopp et al., 1985) have been reported. Recently, Fleeker (1987) described two direct enzyme-linked immunosorbent assays (ELISA) developed for the detection of 2,4-D in water. To date, no immunoassays have been reported for picloram. The following report describes the development of an indirect ELISA procedure for 2,4-D and simple RIA procedures for 2,4-D and piclo-

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ram detection in river water and urine samples without prior cleanup procedures.

MATERIALS AND METHODS

Chemicals and Materials. The analytical standard of picloram and the [2,6-^{14}C]picloram (sp act. 264 MBq/mmol) were provided by the Dow Chemical Co., Midland, MI. The analytical standard of 2,4-D along with *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (CMC), isobutyl chloroformate, triethylamine, bovine serum albumin (BSA), rabbit serum albumin (RSA), goat antirabbit phosphatase, Sigma 104 phosphatase substrate tablets, Tween 20 (poly(oxyethylene)sorbitan monolaurate), Freund's complete adjuvant, and Freund's incomplete adjuvant were obtained from Sigma Chemical Co., St. Louis, MO. The ^{14}C-labeled 2,4-D [(2,4-dichlorophenoxy)[2-^{14}C]acetic acid; sp act. 11.6 GBq/mmol] was obtained from Amersham/Searle, Don Mills, ON. Aquasol 2 and [2-^{3}H]glycine (sp act. 1609.5 GBq/mmol) were obtained from New England Nuclear Research Products, Boston, MA. Diethanolamine was obtained from Fisher Scientific Ltd., Don Mills, ON.

Caution: Precaution is advised when handling the herbicides mentioned in this paper; avoid contact, wear protective clothing, avoid inhalation, and work in a fume hood.

Instruments. The optical density of microtiter plate well content was read on a Bio-Rad Model 2550 EIA reader. Liquid scintillation spectroscopy was performed on a Packard Tri-Carb 460C liquid scintillation system.

Buffers. Phosphate-buffered saline (PBS) contained 8.00 g of NaCl, 0.20 g of KH_2PO_4, 2.90 g of $Na_3PO_4{\cdot}12H_2O$, and 0.50 g of KCl/L of distilled water. The pH was adjusted to 7.4 with 1 M HCl. PBS–Tween washing solution was prepared by adding 0.5 mL of Tween 20/L of PBS. Diethanolamine buffer contained 100 mL of diethanolamine/L of distilled water. The pH was adjusted to 9.8 with 1 M HCl.

Water and Urine Samples. River water was collected from the Speed River, Guelph, Ontario. The water was filtered through Whatman No. 1 filter paper and stored at 4 °C until time of analysis. Human urine was collected from a male donor over a 24-h period, pooled, and stored at 4 °C. Water and urine samples were fortified with an ethanolic solution of analytical standard of 2,4-D or picloram to achieve final sample concentrations within the working range of the immunoassays.

Preparation of Immunogens. Picloram and 2,4-D were conjugated to BSA as described by Fleeker (1987). Equimolar amounts of [^{14}C]picloram (46 mg, 45.5 Bq), NHS (22 mg), and DCC (39 mg) were dissolved in the sequence given in 2.5 mL of dioxane. The solution was allowed to stand at room temperature for approximately 18 h at which time it was filtered to remove the precipitate. The filtrate was evaporated to dryness on a rotary evaporator under vacuum at 35 °C. A solution of BSA (500 mg) dissolved in 3 mL of 0.10 M borate buffer (pH 9) was added to the residue, and the mixture was agitated gently for 1 h at room temperature. The resulting solution was dialyzed against several changes of deionized water over 36 h at 4 °C and lyophilized. The procedure was repeated with [^{14}C]2,4-D (42 mg, 45.5 Bq) in place of picloram. The amount of herbicide bound to BSA was estimated by measuring ^{14}C present in weighed portions of product dissolved in PBS. Approximately 20 and 15 molecules of picloram and 2,4-D, respectively, were bound per BSA molecule.

Antisera. New Zealand white rabbits were injected subcutaneously with an emulsion consisting of 0.5–1.0 mg of immunogen dissolved in 0.5 mL of PBS and an equal volume of Freund's complete adjuvant. The injections were repeated 3, 6, and 10 days after the initial injection, substituting Freund's incomplete adjuvant for complete adjuvant. A booster injection was given 1 month after the initial injection and was repeated at monthly intervals thereafter. The rabbits were bled for antibody titer determinations 10 days after each boost. Antisera for 2,4-D or picloram immunoassay development were prepared from a single bleed in each case.

Preparation of Coating Antigen. To a solution of 50 mg of 2,4-D (0.23 mmol) in 2 mL of dioxane was added 50 mg of CMC (0.12 mmol). The solution was stirred for 2 h at room temperature. RSA (50 mg) was dissolved in 6 mL of 0.1 M borate buffer (pH 9). The 2,4-D solution was added dropwise to the RSA solution over a period of 15 min. The mixture was stirred for 18 h at 4 °C and dialyzed against several changes of deionized water.

Preparation of Radiolabels. The mixed anhydride of 2,4-D was prepared by adding 2,4-D (6 mg), triethylamine (5 μL), and isobutyl chloroformate (5 μL) in the sequence given to 500 μL of dioxane. A portion of the mixed-anhydride solution (100 μL) was added to a solution of 100 μL of [^{3}H]glycine (0.1 mCi), 100 μL of dioxane, 100 μL of distilled water, and 2 μL of 2 M NaOH. After 1 h, an additional 2 μL of NaOH was added. The reaction was allowed to proceed for a total of 4 h at room temperature.

The 2,4-D–[^{3}H]glycine conjugate was isolated and purified by TLC. The reaction mixture, [^{3}H]glycine, and 2,4-D were spotted on a silica gel plate (Whatman K5F). The plate was developed in a diethyl ether–petroleum ether–formic acid (70:30:2, v/v/v) solvent system to a 10-cm solvent front. The 2,4-D standard and the unreacted mixed anhydride of 2,4-D were visualized under UV light (R_f 0.82). Fractions of the plate were scraped, eluted with 90% EtOH, and assayed for radioactivity. Three fractions contained appreciable amounts of radioactivity: R_f 0.00 (corresponding to [^{3}H]glycine), R_f 0.47, and R_f 0.76. The fractions were assayed for binding in an RIA using antisera known to have anti-2,4-D activity. Only the fraction corresponding to R_f 0.47 showed binding. The binding also was shown to be competitively inhibited with free 2,4-D. The fraction chromatographing to R_f 0.47 was therefore assumed to be the 2,4-D–[^{3}H]glycine conjugate.

The mixed-anhydride reaction was repeated with picloram in place of 2,4-D. The picloram–[^{3}H]glycine conjugate was isolated and purified by TLC as described above with the exception that a solvent system of 60:40:2 diethyl ether–petroleum ether–formic acid (v/v/v) was used for optimum separation.

ELISA Procedure. 1. Microtiter plates were coated by adding 100 μL of coating antigen/well (0.023 mg of protein/mL) and incubating for 30 min at room temperature.

2. The plate was emptied and washed once with PBS–Tween (200 μL/well).

3. Unoccupied sites on the polystyrene well surface were blocked by treating with a 5% (w/v) solution of powdered milk in PBS (200 μL/well) for 30 min at room temperature.

4. The plate was emptied and washed two times with PBS–Tween as above.

5. Diluted antiserum (1:1000) was preincubated (15 min) with herbicide standard and sample solutions. Aliquots (100 μL/well) of the preincubated mixture were transferred to the wells of the microtiter plate and incubated for 1 h at room temperature. One column of the plate received no coating and no herbicide in order to determine nonspecific binding while another column received diluted antisera only to determine the maximum absorbance reading (B_0).

6. The plate was emptied and washed two times with PBS–Tween.

7. Goat antirabbit phosphatase conjugate diluted in PBS (1:5000) was added (100 μL/well) to the plate. The plate was incubated for 30 min at room temperature.

8. The plate was emptied and washed two times with PBS–Tween.

9. Substrate (1 tablet/5 mL of diethanolamine buffer) was added to the plate (100 μL/well). Color was allowed to develop for 1 h or until a reading of 0.6 to 0.8 AU was obtained.

10. Absorbance of each well was measured at 405 nm. Absorbance of the standards corrected for nonspecific binding was divided by B_0 (also corrected for nonspecific binding). This value was plotted against the log of herbicide concentration (ng/mL) to construct a standard curve. Concentrations of unknowns were calculated on the basis of the standard curve.

RIA Procedure. The following RIA procedure is a modified version of that described by Weiler et al. (1986).

1. Into 1.5-mL microcentrifuge tubes (Fisher Scientific, Don Mills, ON) was transferred 100 μL of standard or sample. Control tubes received 100 μL of nonfortified sample solution.

2. Incubation mix (300 μL/tube) consisting of 1 part deionized water, 1 part inert serum, 12 parts PBS, and sufficient radiolabel to yield 10 000 cpm per assay was added to each tube.

3. Antisera diluted in PBS (1:100 for 2,4-D antisera, 1:600 for picloram antisera) was added to the tubes (100 μL/tube). One set of control tubes did not receive antisera for determination of nonspecific binding, and a second set of control tubes received

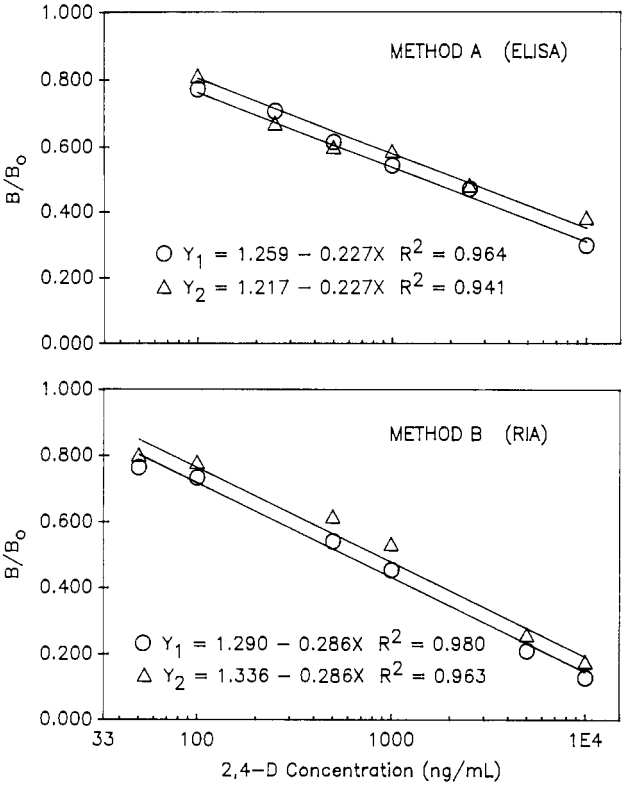

Figure 1. Standard curves for duplicate runs for the determination of 2,4-D by method A (ELISA) and method B (RIA). Each point represents the mean of four or five determinations.

antisera only for maximum binding of radiolabel (B_0).

4. The contents of the tubes were mixed thoroughly on a Vortex mixer followed by a 2-h incubation at 4 °C.

5. The antibody-bound radiolabel fraction was precipitated by adding 0.5 mL of a 90% saturated $(NH_4)_2SO_4$ solution, mixing, and incubating for 1 h at 4 °C.

6. The precipitate was centrifuged (12000*g*) for 5 min, and the supernatant was discarded. The pellet was washed once with a 0.5-mL portion of a 50% saturated $(NH_4)_2SO_4$ solution. The tubes were recentrifuged, and the supernatant was discarded. The pellet was dissolved in two 300-μL aliquots of deionized water, which were transferred to 6-mL scintillation vials. Each vial received 4 mL of scintillation cocktail (Aquasol 2).

7. The scintillation vials were assayed for radioactivity. All results were corrected for nonspecific binding. Values for standards were divided by B_0 and were plotted against the log of the herbicide concentration (ng/mL). The quantity of the herbicide in the unknown sample was calculated on the basis of the standard curve.

RESULTS AND DISCUSSION

A linear relation between the log of 2,4-D concentration and relative absorbance (B/B_0) was found in the range 100–10 000 ng/mL for the indirect ELISA procedure (Figure 1A). A similar relationship was shown between 50 and 10 000 ng/mL of 2,4-D (Figure 1B) and between 50 and 5000 ng/mL of picloram (Figure 2) for the RIA procedures. Statistical analyses showed that, for each method, the slope of the standard curve remained constant between experimental runs while the elevation of the line was subject to small variations. The coefficient of variation (cv) within a run was 7% or less for 2,4-D determined by the indirect ELISA method, 9% or less for 2,4-D by the RIA method, and 3% or less for picloram determined by the RIA method.

Recoveries from fortified river water and human urine samples determined by the immunoassays were good, with mean overall recoveries varying from 82% to 110% (Table I). The range of concentrations over which 2,4-D and

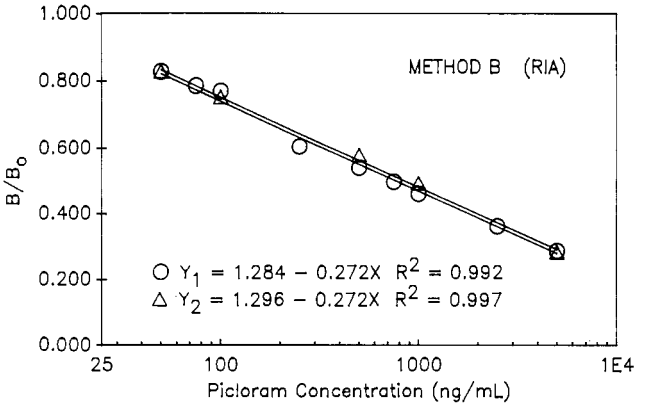

Figure 2. Standard curves for duplicate runs for the determination of picloram by RIA. Each point represents the mean of four or five determinations.

Table I. Recovery of Analyte from River Water and Human Urine Samples As Determined by RIA or Indirect ELISA

method	analyte	amt analyte added, μg/mL	recovery[a] river water	recovery[a] human urine
RIA	2,4-D	0.25	0.21 ± 0.04 (4)	0.25 ± 0.01 (8)
		2.50	2.35 ± 0.04 (4)	2.65 ± 0.15 (7)
ELISA	2,4-D	0.25	0.29 ± 0.06 (2)	0.25 ± 0.03 (9)
		0.75	ND[b]	0.90 ± 0.13 (7)
		2.50	2.44 ± 0.92 (2)	ND
RIA	picloram	0.25	0.25 ± 0.03 (6)	0.19 ± 0.04 (6)
		2.50	2.60 ± 0.19 (6)	2.22 ± 0.35 (6)

[a] Mean recovery: micrograms per milliliter ± SE (number of determinations). [b] ND = not determined.

picloram were accurately quantitated with no sample cleanup corresponds with levels found in urine in applicator exposure studies conducted by Libich et al. (1984) as well as with levels reported from environmental fate studies conducted by Thompson et al. (1984) and Hall et al. (1987). With a concentration step, such as the one described by Fleeker (1987) using disposable reversed-phase preparative chromatography columns (octadecylsilane-bonded phase packing; C_{18}), the immunoassays also could be applied to well water contamination studies where a lower limit of detection is required (Frank et al., 1987).

To determine the specificity of the antisera for 2,4-D, a RIA was conducted whereby binding of the 2,4-D–[^{3}H]glycine radiolabel was inhibited with structurally similar herbicides at concentrations up to 10 000 ng/mL. The specificity of the picloram antisera was determined in a similar manner. The results indicated that 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid], MCPA [(4-chloro-2-methylphenoxy)acetic acid], and dichlorprop [(±)-2-(2,4-dichlorophenoxy)propanoic acid] cross-reacted with the 2,4-D antisera to some extent (Table II). The antisera were 6 times more specific for 2,4-D than for the strongest competitor, MCPA. None of the related herbicides tested were able to inhibit binding of the picloram radiolabel by 50%. The lack of specificity of the picloram antisera for 2,4-D is particularly important since picloram is sold commercially as a mixture with 2,4-D.

The RIA methods reported here incorporate a novel radiolabel. Herbicides labeled with ^{14}C are easily obtained but do not lend themselves to sensitive and accurate immunoassay work because of low specific activities (Hammock and Mumma, 1980). Radioimmunoassays utilizing high specific activity radiolabels such as [^{3}H]2,4-D (Knopp et al., 1985) or a [^{125}I]2,4-D derivative (Rinder and Fleeker, 1981) have given good results. Covalently linking the herbicide molecule with [^{3}H]glycine yields a radiolabel with

Table II. Specificity of Antisera for 2,4-D or Picloram Compared to Some Structurally Similar Herbicides Determined by the RIA Method

antisera	compound[a]	amt compd for 50% inhibn in binding of radiolabel, ng/mL
2,4-D	2,4-D	560
	MCPA	3600
	2,4,5-T	5000
	dichlorprop	10000
	mecoprop	>10000
	dicamba	>10000
picloram	picloram	760
	clopyralid	>10000
	triclopyr	>10000
	2,4-D	>10000

[a] Key: 2,4-D, (2,4-dichlorophenoxy)acetic acid; MCPA, (4-chloro-2-methylphenoxy)acetic acid; 2,4,5-T, (2,4,5-trichlorophenoxy)acetic acid; dichloroprop, (±)-2-(2,4-dichlorophenoxy)propanoic acid; mecoprop, (±)-2-(4-chlorophenoxy)propanoic acid; dicamba, 3,6-dichloro-2-methoxybenzoic acid; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; clopyralid, 3,6-dichloro-2-pyridinecarboxylic acid; triclopy, [(3,5,6-trichloro-2-pyridinyl)oxy]-acetic acid.

high specific activity without the expense of purchasing a custom-synthesized tritiated herbicide or the health hazards connected with iodated radiolabels.

In practice, the RIA was a much simpler procedure requiring fewer steps to complete the assay. The formation of the antibody–antigen complex depends on a combination of weak noncovalent bonds including hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic bonds. Likewise, the passive binding of coating antigen to the microtiter plate well surface depends on these same forces. A successful indirect ELISA requires the formation of these weak bonds at three separate sites: (i) between the plate surface and the coating antigen, (ii) between the coating antigen and the antibody, and (iii) between the antibody and the goat anti-rabbit–enzyme complex. In comparison, the RIA relies only on the formation of the antibody–antigen complex. Therefore, the RIA was the preferred assay procedure since there is less possibility for the introduction of experimental error. It is our opinion that, for pesticide determinations, a direct ELISA with monoclonal antibodies specific for the particular herbicide would provide a more simple and reliable assay compared to the polyclonal indirect ELISA system.

The immunoassays reported here could be incorporated on a routine basis in most laboratories to serve one of two functions. The assays could be used as a rapid, inexpensive method for herbicide quantitation with no sample cleanup. Alternatively, they may be implemented as a preliminary screen to rank samples for follow-up determination by gas chromatography. In either function, the immunoassays represent savings in time, labor, and materials.

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Registry No. 2,4-D, 94-75-7; picloram, 1918-02-1; water, 7732-18-5.

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