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A Radioimmunoassay to Screen for 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid in Surface Water

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Monitoring of environmental samples for pesticides has become a routine duty for some laboratories. Present-day methods often use liquid or gas chromatography (GC). Immunological techniques, such as the radioimmunoassay (RIA), offer an attractive alternative in some cases (ERCEGOVITCH 1971). The RIA is particularly useful for the routine analysis of a large number of samples.

The present method of choice for determination of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) involves extraction, derivatization and GC (MARQUARDT et al. 1964; U.S. ENVIRONMENTAL PROTECTION AGENCY 1979). Described herein is a screening method which uses an RIA to detect the presence of these two herbicides in surface and ground water.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co., St. Louis MO (no. 7638). Freund's adjuvant was purchased from Calbiochem, San Diego, CA. Standards of 2,4-D, 2,4,5-T and 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) were supplied by the U.S. Environmental Protection Agency, Research Triangle Park, NC. Other compounds used were recrystallized from the appropriate solvent. [Acetic-2-^{14}C]2,4-D was purchased from The Radiochemical Centre, Amersham, England. Na^{125}I was purchased from New England Nuclear, Boston, MA (NEZ-033). [^{36}Cl]2,4,5-T was prepared from 2,4-D by the method of MIKULSKI AND ECKSTEIN (1959).

Preparation of immunogen. The preparation of 2,4-dichloro-5-aminophenoxyacetic acid (5-NH_2-2,4-D) from 2,4-D has been described by DRINKWINE (1979). Radioactive 5-NH_2-2,4-D was prepared from [acetic-2-^{14}C]2,4-D. Sodium nitrite (28 mg), in 1 mL of cold water, was added slowly to 97 mg [^{14}C]5-NH_2-2,4-D in 5 mL 0.8 M HCl. After 5 min, the diazonium salt was slowly added to a stirred and cooled solution of 710 mg BSA in 35 mL water and 1 mL 1 N NaOH. Additional alkali was added as required to maintain a deep red color in the reaction milieu. The solution was stirred and cooled an additional hour, then the pH adjusted to 7 with HCl. The clear-red solution was dialyzed against four, 2-L changes of water and freeze-dried. Five moles

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of [^{14}C]5-NH_2-2,4-D were incorporated per mole of BSA as determined by isotope incorporation.

Antisera. Three New Zealand rabbits were injected intramuscularly on the flanks with 0.5 mL of a solution of 5 mg immunogen in 1 mL of 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.2, emulsified with an equal volume of Freund's adjuvant. The animals were immunized again 10, 21 and 71 days later. Serum was prepared from blood collected from the marginal ear vein. Bleeding was done at 7 to 10 day intervals. Serum was stored at -20°C until needed.

Preparation of (2,4-dichloro-5-carboxymethoxyphenyl-4'-hydroxyphenyl diazene. Sodium nitrite (0.5 g) in 5 mL water was slowly added to a stirred solution of 1.2 g of 5-NH_2-2,4-D in 5 mL 37% HCl at 0°C. The resulting diazonium salt was added in a thin stream to 0.47 g of phenol in 25 mL cold 10% NaOH. The mixture was stirred overnight at 0-4°C. The pH was adjusted to 2 with 6 M HCl and the precipitate collected by suction filtration. The solid was recrystallized twice from aqueous methanol. The yield was 25% at this point. The product was chromatographed on a silica gel TLC plate with diethyl ether-hexane-formic acid (70:30:2). Several minor and one major bands were detected. The major band was isolated by preparative thin-layer chromatography (R_f 0.5), resulting in orange crystals, m.p. 250°C. A solution of this compound, when allowed to stand several hours and rechromatographed, gave two bands, which suggests an equilibrium between the cis and trans isomers of the diazene. The NMR spectrum was consistent with a para-substituted phenol.

Preparation of ^{125}I-labeled ligand. Iodination was carried out by adding the following reagents, in the sequence given, to 1.0 mCi of Na^{125}I in 10 µL of 0.1 M NaOH: 81 ng of the diazene in 10 µL of 0.25 M sodium phosphate, pH 7.5, and 50 µg chloramine T in 10 µL 0.05 M phosphate, pH 7.5. Two min after the last addition 120 µg of sodium metabisulfite in 100 µL 0.05 M phosphate, pH 7.5, was added. After two min, the solution was rinsed into a tube with three 100-µL portions of KI (1 mg/mL). The solution was acidified with 20 µL of 6 M HCl and extracted three times with 1-mL portions of diethyl ether. The combined ether extracts were taken to dryness in the hood with a stream of N_2. The residue was taken up in acetone and the solution applied to a thin-layer plate of silica gel G. The plate was developed with toluene-ethyl acetate (1:1). The solvent was allowed to move 12 cm beyond the origin. The origin was scraped off the plate and the silica gel extracted with methanol. The methanol extract was applied to a second plate which was developed with diethyl ether-hexane-formic acid (70:30:2). The area of the chromatogram with an R_f corresponding to the unlabeled diazene was removed and extracted with methanol. The methanolic solution was stored at 4°C. Aliquots were diluted with assay buffer for use in the determination.

Extraction of 2,4-D and 2,4,5-T from water. River water was

first clarified by centrifugation. A 10-mL aliquot was acidified with 0.5 mL of 0.25 M HCl. A C_{18} cartridge (SEP-PAK C_{18}, Waters Associates, Inc., Milford, MA) was prewashed with 3 mL methanol, then twice with 3-mL portions of distilled water. The acidified sample was forced through the cartridge at approximately 10 mL/min, followed by a rinse of 3 mL of distilled water. The eluates were discarded. The 2,4-D and 2,4,5-T retained by the cartridge were eluted with 4 mL methanol. The eluate was collected in the tube used for the RIA incubation and was taken to dryness with a stream of N_2 gas. The residue at this point may be taken up in 100 µL of assay buffer and used directly in the assay.

Assay buffer. The assay buffer contained 120 mM sodium borate, 150 mM NaCl and 0.5% Triton X-100, pH 8.2.

Staphylococcal-protein A immunoabsorbant. A commercial preparation of formalinized, heat-killed and freeze-dried whole cells of Staphylococcus aureus Cowan strain I (IGSORB, The Enzyme Center, Inc., Boston, MA) was used to remove immunoglobulins from solution in the assay procedure. The cells were reconstituted as a 10% suspension. The fresh suspension was divided into 5-mL aliquots, which were frozen in acetone-dry ice and stored at -20°C until used.

Immunoassay protocol. Sera were diluted in assay buffer. A solution of ^{125}I-labeled ligand (100 µL), containing 15,000 cpm of radioactivity, was added to 100 µL of analyte solution. The resulting solution was treated with 100 µL of appropriately diluted serum and incubated one hour at 25°C. Staphylococcus cells (100 µL) were added and after 10 min the mixture was centrifuged (2000 x g, 10 min, 4°C). The supernatant was discarded. The pellet was suspended in 2 mL of assay buffer, the mixture centrifuged and the supernatant discarded. The pellet was assayed for radioactivity in a gamma scintillation counter.

RESULTS AND DISCUSSION

Sera from the three test animals were examined for immunoreactivity with 2,4-D, 2,4,5-T and related compounds (Table 1). Three plant metabolites of 2,4-D were tested; 2,4-dichlorophenol, 2,3-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,3-D) and 2,5-dichloro-5-hydroxyphenoxyacetic acid (4-OH-2,5-D). All displayed low immunoreactivity. The herbicides 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-DB, and 2-(2,4,5-trichlorophenoxy)-propionic acid (silvex) had greater immunoreactivity than the metabolites, but to an extent which was less than 10% of 2,4-D. Serum from rabbit 3 was used to develop the assay. The serum desired for this assay was that which displayed high and similar immunoreactivity with 2,4-D and 2,4,5-T, and gave the lowest reactivity with other related compounds.

2,4,5-T showed greater immunoreactivity with the sera than 2,4-D (Table 1). This was probably due to the structure of the

TABLE 1. Immunoreactivity of 2,4-D, 2,4,5-T and related compounds as measured by displacement of the ^{125}I-labeled ligand from antibody.

Compound	Nmoles required for 50% displacement[a]		
	Rabbit 1	Rabbit 2	Rabbit 3
2,4-D	0.059	0.059	0.029
2,4,5-T	0.013	0.0082	0.0055
MCPA	1.0	0.18	1.2
2,4-DB	>12	4.4	>12
Silvex	0.93	0.13	0.37
2,4-Dichlorophenol	>18	>18	>18
4-OH-2,5-D	1.1	0.76	1.26
4-OH-2,3-D	4.6	2.5	13

[a] The quantity of the compound in 0.1 mL necessary to inhibit binding of 50% of the labeled ligand in the immunoassay.

hapten attached to BSA. The nitrogen derived from the amino group of 5-NH_2-2,4-D appears to be immunologically recognized as a "surrogate chlorine" atom. Thus, the hapten has greater structural similarity with 2,4,5-T than 2,4-D. This phenomenon has been observed in other immunogens by Luster, et al. (1980). Should serum with a greater specificity for 2,4-D than 2,4,5,-T be desired, a hapten derived from 2-chloro-4-aminophenoxyacetic acid could be used.

The C_{18} cartridges were used to extract and concentrate the herbicides from water. The recoveries of 2,4-D from water were similar and reproducible over a range of 0.01 to 10 µg/10 mL of sample volume (Table 2). Recovery was verified with radioactive 2,4,5-T and 2,4-D. Repeated use of a cartridge resulted in a 1-3% decrease in recovery with each reuse.

TABLE 2. Recovery of [^{14}C]2,4-D and [^{36}Cl]2,4,5-T from 10 mL river water using C_{18} cartridges.

Compound added	Weight (µg)	Number of determinations	Percent of radioactivity recovered (mean ± SD)
[^{14}C]2,4-D	0.01	8	97.2 ± 7.9
[^{14}C]2,4-D	0.1	12	96.9 ± 4.4
[^{14}C]2,4-D	1.0	12	92.5 ± 1.9
[^{14}C]2,4-D	10.0	12	90.2 ± 1.9
[^{36}Cl]2,4,5-T	1.0	4	94.3 ± 1.5

The cartridges served as a convenient method of concentrating the herbicides, thus increasing the sensitivity of the method. The standard curve for 2,4-D was essentially linear in the range

of 1 to 10 ng/100 µL (Figure 1). The C_{18} cartridges permitted the concentration of 2,4-D ten-fold so that 1 ng in 10 mL (0.1 ng/mL) of the original aqueous sample could easily be detected. A similar response from 2,4,5-T, assuming similar extraction efficiency, would require 0.02 mg/mL in the sample before concentration.

The precipitation of antibody with Staphylococcus cells offers several advantages over other methods which separate antibody from unbound labeled ligand (KESSLER 1975; RUCH AND KNIGHT 1980). Among the advantages are simplicity and rapidity. Protein A, linked to the bacterial cell wall, binds specifically and strongly to the Fc portion of immunoglobulins from most mammalian species, including the rabbit (KRONVALL et al. 1970). However, the binding is not as strong for a few species such as the goat.

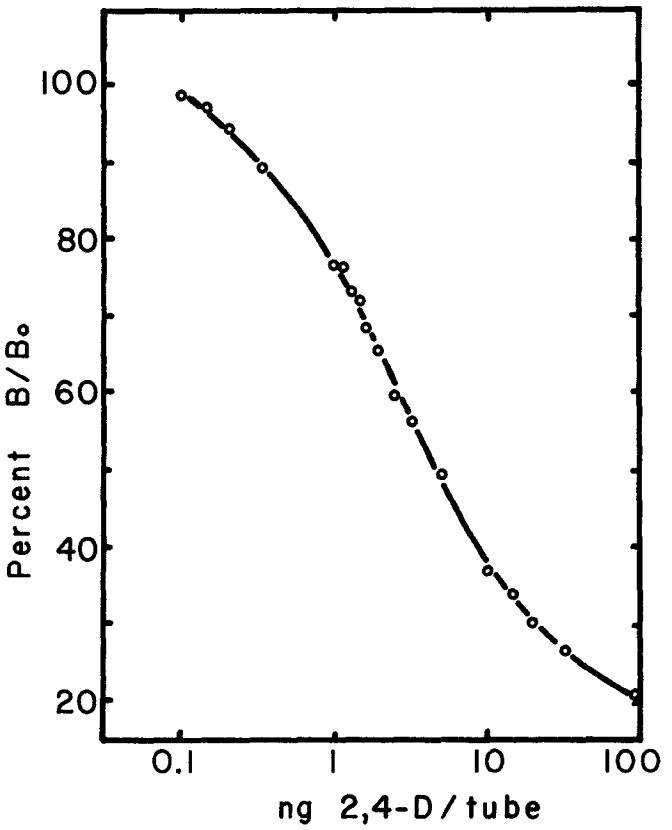

Figure 1. Standard curve for the RIA of 2,4-D. Each point represents an average of three to four determinations.

The assay as described here is a screening method for 2,4-D and 2,4,5-T in surface or ground water. The method as described cannot distinguish between the two compounds. The technique would be used for screening a large number of samples on a routine basis. A sample giving a positive response, as arbitrarily established by the analyzer, could subsequently be analyzed by GC to determine the identity and amount of the chlorinated phenoxyacetic acid present.

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