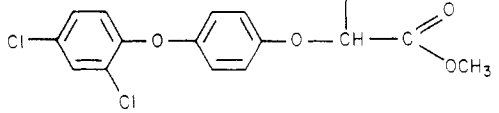
Enzyme Immunoassay and Fluoroimmunoassay for the Herbicide Diclofop-methyl

Margit Schwalbe, Erich Dorn, and Klaus Beyermann*

This paper describes the development of enzyme immunoassay and fluoroimmunoassay for the herbicide diclofop-methyl [methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate]. To produce antibodies against the herbicide, rabbits were immunized with a conjugate of diclofop acid and bovine serum albumin, which was prepared by use of a water-soluble carbodiimide. The resulting antisera showed a high specificity toward the (dichlorophenoxy)phenoxy moiety of the herbicide, which was demonstrated by the determination of the cross-reactions of structurally related compounds. The detection limits for diclofop-methyl in the fluoroimmunoassay and enzyme immunoassay were 45 and 23 ng/mL, respectively. Both methods permit the quantification of the herbicide in soil, urine, serum, wheat, soybeans, and sugar beets, facilitating reduced sample cleanup. The values obtained by the immunoassay procedures and by gas chromatography or liquid scintillation counting of radioactivity were identical within the limit of errors of the procedures.

In the fields of clinical chemistry and endocrinology, immunochemical methods offer many advantages for the analysis of drugs or biochemicals including sensitivity, specificity, and speed of analysis. In pesticide analysis, however, they are rarely applied. Residue analyses of pesticides have mainly been based on conventional techniques such as chromatographic or colorimetric procedures and mass spectrometry. These methods are often time consuming and expensive. Moreover, they require a drastic sample cleanup. Immunological procedures allow to determine low concentrations of pesticides by taking advantage of the specificity of the antigen–antibody reaction. In Table I the immunoassays so far described for the determination of pesticides are listed. Diclofop-methyl is a selective herbicide for the control of weed grasses (Nestler, 1982), having the structural formula



methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate

The immunoassays for the determination of diclofop-methyl show some advantage over conventional procedures in routine residue analysis. Radioactivity is the mostly used signal to be measured in immunoassays. Although this version is very sensitive, it has certain disadvantages: the use of radioactive tracers requires special permission of authorities and expensive equipment. Moreover, the practicability of an assay is limited by the half-life of isotopes. In the practice of routine residue monitoring, easy, quick, and cheap methods are needed. Because of these reasons, we focused on nonisotopic labels, such as enzymes or fluorescent probes.

MATERIALS AND METHODS

Reagents and Equipment. Diclofop-methyl, dichlofop, ^{14}C-labeled diclofop-methyl (specific activity 733 MBq/g), and the compounds tested for their cross-reactivities were obtained from Hoechst AG (Frankfurt/Main, West Germany). Bovine serum albumin (BSA) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluenesulfonate (reinst) were purchased from Serva Feinbiochemica (Heidelberg, West Germany). Polyethylene glycol 6000 (Zur Synthese, average molar mass 5000–7000, melting point 55–62 °C) was obtained from Merck (Darmstadt, West Germany) and used without further purification. Fluorescein amine and γ-globulin from bovine serum (Cohn fraction II, 98%) were obtained from Fluka AG (Buchs, Switzerland). Goat antibody to rabbit γ-globulin (lyophilized) was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and horseradish peroxidase from Boehringer Mannheim GmbH (West Germany). Sephadex G-50 (fine, particle size 20–80 μm) was from Pharmacia Fine Chemicals (Uppsala, Sweden). All the other chemicals used were obtained from Merck (Darmstadt, West Germany).

Table I. List of Immunoassays for the Analysis of Pesticides

pesticide	literature
dieldrin, aldrin	Langone and Van Vunakis (1975)
2-aminobenzimidazole (degradation product of benomyl)	Lukens et al. (1977)
S-bioallethrin	Wing et al. (1978); Wing and Hammock (1979)
paraquat	Levitt (1977, 1979); Fatori and Hunter (1980)
2,4-D, 2,4,5-T	Rinder and Fleeker (1981)
benomyl	Newsome and Shields (1981)
parathion	Ercegovich et al. (1981); Vallejo et al. (1982)
paraoxon	Hunter and Lenz (1982)
diflubenzuron, BAY SIR 8514	Wie et al. (1982); Wie and Hammock (1982)

For fluorometry an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD) equipped with a xenon arc lamp source and monochromator slit widths of 2 mm for both excitation and emission was used. All spectrophotometric measurements were performed with a Zeiss PMQ II spectrophotometer. The ^{14}C-labeled compounds were determined by using an ICN Tracerlab (Köln, West Germany), Zinsser polyvials (27 × 60 mm; Zinsser, Frankfurt/Main, West Germany), and 10 mL of scintillation cocktail (XAA 8; Riedel-de Haen AG, Seelze, West Germany).

For gas chromatography a Varian gas chromatograph, Model 3700, equipped with a ^{63}Ni electron-capture detector operated at 280 °C was used. The glass column (2 m × 2 mm i.d.) was packed with 3% silicone OV-225 on Chromosorb W-HP, 100–120 mesh. The carrier gas was nitrogen at a flow rate of 40 psig. With a column temperature of 230 °C and an injector temperature of 260 °C,

Johannes Gutenberg-Universität, Institut für Anorganische und Analytische Chemie, 6500 Mainz, West Germany (K.B.), and Analytisches Labor, Hoechst Aktiengesellschaft, 6230 Frankfurt/Main 80, West Germany (M.S. and E.D.).

0021-8561/84/1432-0734$01.50/0

diclofop-methyl has a retention time of 14.0 min.

Preparation of Immunogen. For the preparation of the immunogen, diclofop acid was covalently linked to bovine serum albumin (BSA) with the aid of the water-soluble 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluenesulfonate (CDI). BSA (200 mg) was dissolved in 10 mL of H_2O and the pH of the solution adjusted to 5.5. CDI (93 mg) was added to the stirred protein solution. Diclofop (37 mg) was dissolved in 2 mL of dimethylformamide and added slowly while maintaining the pH at 5.5. Fifteen minutes after the addition of diclofop another 42-mg portion of CDI was added. Stirring was continued for 15 min at room temperature. The solution was then dialyzed for 48 h against several changes of distilled water. The product was then freeze-dried. From the UV absorption at 279 nm, it was calculated that the immunogen contained 17 molecules of diclofop per molecule of BSA.

Immunization. The immunization was performed at Behringwerke AG (Marburg, West Germany). Five rabbits were used to obtain antibodies against the BSA–diclofop conjugate. All animals received the same treatment. At the beginning of the immunization period each rabbit got a sc injection of 0.75 mg of immunogen emulsified in 2 mL of saline solution and 2 mL of Freud's complete adjuvant at different sites in the vicinity of their lymph nodes. The immunization was continued from the second to the fifth day by daily iv administration of 0.1 mg of the conjugate in 3 mL of a 0.05% Aerosil solution (Aerosil No. 130, Fa. Degussa, Hanau, West Germany). The procedure was repeated in the third, fifth, seventh, and twelfth week in the same manner described above. Further booster injections of 0.1 mg of the immunogen in 3 mL of 0.05% Aerosil solution were given at intervals of 3–4 weeks during the period of 1 year. The antisera were taken by plasmapheresis. The first amounts of antiserum were withdrawn 36 days after the start of the immunization. Further withdrawals were taken after the booster injections. The rabbits were killed by bleeding after 58 weeks of treatment.

Characterization of Antisera. For the first characterization of the antiserum affinity, binding tests with ^{14}C-labeled diclofop-methyl were performed as follows: A solution of ^{14}C-labeled diclofop-methyl (0.1 mL; 50 ng; 2200 dpm) in 0.05 M phosphate buffer, pH 7.4, 0.02 mL of the antiserum sample, and 0.38 mL of the phosphate buffer, pH 7.4, were incubated in polystyrene tubes (12 × 75 mm) at 4 °C for 24 h. After the addition of 0.5 mL of saturated ammonium sulfate solution, the tubes were vortex-mixed and centrifuged for 15 min at 3000 rpm (1500*g*). Five-tenths milliliter of the supernatant fluid was transferred to a scintillation vial. After the addition of 10 mL of scintillation cocktail (XAA 8), radioactivity was measured for 10 min. It was carefully checked that no phase separation occurred when measuring the aqueous solutions with ammonium sulfate by liquid scintillation counting. The measurement of the supernatant fluid was adequate for determining titer and average affinity of antisera. If a sensitive radioimmunoassay shall be developed, the pellet should be assayed for radioactivity. The determined amount of radioactivity is inversely proportional to the affinity of the antiserum. All samples were performed in duplicate. Unspecific effects could be controlled by the use of normal rabbit serum.

Synthesis of Fluorescein Amine Labeled Diclofop. Fluorescein amine labeled diclofop was synthesized for the use as tracer in the fluoroimmunoassay. Diclofop acid (33 mg) was dissolved in 10 mL of acetone. After the addition of 15 μL of triethylamine, the solution was cooled to −10 °C. Isobutyl chloroformate (13 μL) was added and the solution was stirred for 30 min in the ice bath. Fluorescein amine (35 mg) dissolved in 6 mL of acetone and cooled in the ice bath was added, and the resulting solution was stirred for 10 min at −10 °C and for 15 h at room temperature. The fluorescein amine labeled diclofop was purified by preparative TLC (silica gel plates 0.5 mm, Fa. Merck, Darmstadt, West Germany; acetone as the developing solvent) and column chromatography on silica gel with acetone as the eluent. Identification of the tracer was performed by mass spectrometry and IR spectroscopy. The stock solution of the tracer in methanol (50 μg/mL) was stored at 4 °C. Working solutions were freshly made before the use by diluting appropriate amounts of the stock solutions with 0.05 M borate buffer, pH 8.0. The fluorescence of the tracer was measured with an excitation wavelength of 495 nm and an emission wavelength of 540 nm. The detection limit of the fluorescent tracer was 5 ng/mL in 0.05 M borate buffer, pH 8.0.

Fluoroimmunoassay. All solutions were made in 0.05 M borate buffer, pH 8.0, and all steps were performed at room temperature. Standard or samples or buffer (0.2 mL), 0.1 mL of the tracer working solution (30 ng), and 0.1 mL of the diluted antiserum were incubated together in polystyrene tubes (12 × 75 mm) for 4 h. After the addition of 0.1 mL of γ-globulin solution (0.75 mg) and 0.5 mL of polyethylene glycol solution (PEG 6000, average molar mass 5000–7000; 25%; w/v), the tubes were vortex-mixed and centrifuged at 3200 rpm (1600*g*) for 20 min. The supernatant fluid was transferred into a quartz cuvette (0.2 × 1 cm); fluorescence intensity was measured with excitation at 495 nm and emission at 540 nm against an analogous reference without the addition of tracer. All samples were run in duplicate; those of the standard curve were run in triplicate. From the measured fluorescence intensity, the amount of tracer bound to the antibodies was calculated. The intensity without addition of antiserum served as value for 0% tracer bound.

Preparation of the Enzyme-Labeled Tracer. For use in the enzyme immunoassay, horseradish peroxidase was coupled to diclofop acid by the mixed anhydride method. Conjugation was performed as follows: ^{14}C-labeled diclofop was obtained by the alkaline saponification of ^{14}C-labeled diclofop-methyl, resulting in a final specific radioactivity of 1.2×10^6 dpm/mg (=20.0 MBq/g). Diclofop from this preparation (12 mg; 1.44×10^7 dpm) and triethylamine (5 μL) were dissolved in 5 mL of dimethyl formamide and cooled to −12 °C. After the addition of isobutyl chloroformate (5 μL), the solution was stirred at −12 °C for 30 min.

Horseradish peroxidase (14.4 mg) was dissolved in 5 mL of H_2O and cooled. One milliliter of the solution of activated diclofop was then slowly added to the stirred enzyme solution. Stirring was continued for 1 h at −12 °C, 1 h at 0 °C, and 15 h at room temperature. The solution was then dialyzed for 48 h against several changes of distilled water. For further purification, the solution was subjected to gel chromatography on Sephadex G-50 with 0.05 M phosphate buffer, pH 7.4. The different fractions were examined by measuring the radioactivity and the UV absorption at 280 and 403 nm. All fractions that contained the enzyme-labeled tracer were pooled, and the resulting solution was used as stock solution for the further experiments. The measurement of radioactivity in the tracer showed that ten molecules of diclofop acid were bound to one molecule of peroxidase. The stock solution was stored at 4 °C and freshly diluted before it was used in the enzyme immunoassay.

Determination of the enzyme activity according to the method described below showed that 70% of the activity of the pure enzyme was preserved in the conjugate.

Measurement of the Enzyme Activity. The activity of the horseradish peroxidase was determined by using the substrates H_2O_2 and 1,2-phenylenediamine as follows: *o*-phenylenediamine (81 mg) was dissolved in 25 mL of 0.1 M sodium citrate buffer, pH 5.0. This solution was stored in the dark. H_2O_2 (25 μL; 30%) was diluted in the same buffer. Five-tenths milliliter of each of the two solutions was added to the sample. Volume was adjusted to 1.5 mL with the cited buffer. After the incubation at room temperature for 1 h, 1 mL of 4 M HCl was added and the resulting color measured in a spectrophotometer at 492 nm against a reference without enzyme. The detection limit was 0.03 ng/mL for the pure enzyme and 0.043 ng/mL for the enzyme-labeled diclofop.

Enzyme Immunoassay. The enzyme immunoassay was performed as follows: For all the incubation steps, 0.05 M phosphate buffer, pH 7.0, was used. Buffer or standard or sample (0.2 mL) and 0.1 mL of the antiserum dilution were incubated for 3 h at 4 °C in glass tubes (20 × 100 mm). After the addition of 0.1 mL of the diluted tracer solution (1.5 ng of enzyme-labeled diclofop), incubation was continued for additional 3 h.

For the separation of the antibody-bound and free fraction of antigen, goat anti-rabbit γ-globulin was reconstituted according to the manufacturer's advice in 0.05 M phosphate buffer, pH 7.0, containing 0.14 M NaCl. The second antibody solution (0.1 mL) and 0.2 mL of polyethylene glycol solution (PEG 6000, average molar mass 5000–7000; 7%; w/v) were added to the samples. The tubes were vortex-mixed and allowed to stand at room temperature for 30 min. After the centrifugation at 3200 rpm (1600*g*) for 20 min, the supernatant fluid was discarded and the precipitate dissolved in 0.5 mL of 0.1 M sodium citrate buffer, pH 5.0. This solution was used to determine the enzyme activity in the antibody-bound fraction according to the description above. Before the measurement of the absorbance, all the samples were centrifuged at 3200 rpm (1600*g*) for 10 min. All the samples were run in duplicate; those of the standard curve were run in triplicate.

Processing of the Biological Samples. Soil (100 g, Schwanheimer Sandboden, Hoechst AG, Frankfurt/Main, West Germany) was placed in a glass column and extracted either with 400 mL of acetonitrile–H_2O (9:1) or with 500 mL of methanol–H_2O (1:1). Sugar beets (20 g) were homogenized 3 times with an Ultra-Turrax with 200 mL of methanol–H_2O (9:1) or 200 mL of acetone. Soybeans (20 g) were treated in the same manner either with 250 mL of methanol–H_2O (8:2) or with 250 mL of chloroform–methanol (1:1). Grains of wheat (10 g) were homogenized 3 times with 300 mL of methanol–H_2O (8:2) and 250 mL of chloroform–methanol (1:1), respectively. Wheat shoots (0.5 g) were treated in the same way with 30 mL of chloroform–methanol (1:1). The organic extracts were evaporated and the residues taken up in 100 mL of buffer, which was 0.05 M borate buffer, pH 8.0, for fluoroimmunoassay and 0.05 M phosphate buffer, pH 7.0, for enzyme immunoassay. Milk (1 mL, 3.5% fat) was extracted according to the procedure described by Tessari and Savage (1980). The final acetonitrile extract was evaporated to dryness and taken up in assay buffer (see above). Normal rabbit serum (1 mL) and human urine (1 mL) were directly diluted with the assay buffers to 4 and 5 mL, respectively. Two-tenths milliliter of the aqueous extracts of the described samples was used in the fluoroimmunoassay. For enzyme immunoassay, the extracts of normal rabbit serum and of sugar beets were 10-fold diluted in order to avoid background activity of peroxidase. To all these final aqueous extracts of the biological samples, known amounts of diclofop-methyl or diclofop were added and the recoveries were determined by the immunoassay procedures. Thus, the fortification of the samples was the last step before performing the immunoassays.

Gas Chromatography and Liquid Scintillation Counting as Reference Methods. In order to control values obtained with fluoroimmunoassay or with enzyme immunoassay, two reference methods were used. Samples that contained ^{14}C-labeled diclofop-methyl or ^{14}C-labeled diclofop acid were examined by liquid scintillation counting after mixing 0.1–2.0 mL of the extracts with 10 mL of the scintillation cocktail XAA 8. Counting efficiencies were determined by adding known amounts of the ^{14}C-labeled diclofop-methyl. From the comparison of the radioactivity with the known specific activity of the ^{14}C-labeled diclofop-methyl, the amount of ester in the sample was calculated. Samples that contained diclofop-methyl were examined by gas chromatography after extracting 1 mL of the aqueous solution 3 times with 6 mL of *n*-hexane and drying the hexane solution with anhydrous sodium sulfate. The hexane extract (2–5 μL) was injected into the gas chromatograph. For the conditions of the gas chromatographic measurement, see Reagents and Equipment. The amounts of diclofop-methyl in the extracts were determined by comparing the peak height with those of the standard curve that was obtained by the use of pure diclofop-methyl.

RESULTS AND DISCUSSION

Production and Characterization of Antisera. A small nonimmunogenic molecule such as diclofop-methyl must be conjugated to a protein in order to elicit antibodies in animals. Therefore, a functional group in the hapten must react with the protein carrier. In diclofop, the free carboxylic group allows the covalent linkage of the acid to BSA via an amide bond. The essential activation step was performed by the aid of a water-soluble carbodiimide. Using the carboxylic function for conjugation, the 2,4-dichlorophenoxy moiety is preserved as the determinant group.

The diclofop-BSA conjugate was effective in inducing the formation of antibodies in rabbits, which could be determined by binding tests with ^{14}C-labeled diclofop-methyl. Thirty-six days after starting the immunization, antibodies were detected in the sera of all animals. In the course of the further immunization, there was a significant increase in the antiserum affinity; therefore, the later samples were able to bind higher amounts of ^{14}C-labeled diclofop-methyl. The maximum titer was achieved after 4 months of immunization. Later, the titer of the antisera remained constant or decreased.

The individual animals showed significant differences in their antisera. With regard to the titer—which is the antiserum dilution that binds 50% of the fluorescent or enzyme-labeled tracer—the anti-diclofop sera from animals 3 and 4 proved to be the most suitable ones and were therefore selected for the further development of the immunoassays. For the use in fluoroimmunoassay, the antiserum samples of animal 3 from the weeks 22–44 after the start of the immunization were pooled. For the enzyme immunoassay the samples of animal 4 from the weeks 14–40 were used. Figure 1 shows the antiserum dilution curves of these pooled antisera. The final dilutions were 1:160 in fluoroimmunoassay and 1:220 in enzyme immunoassay, respectively.

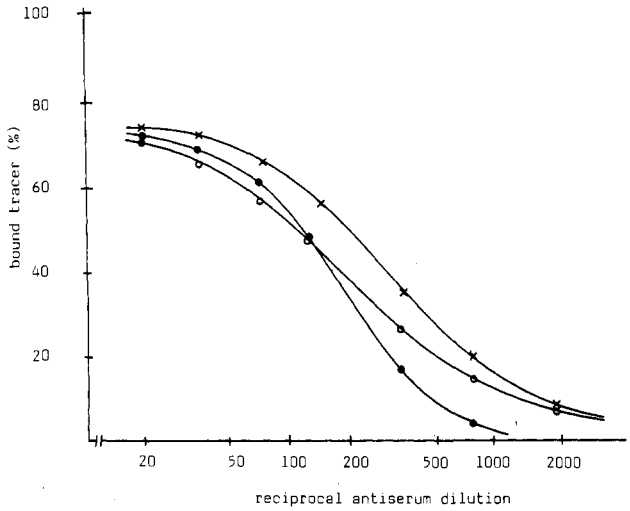


Figure 1. Antiserum dilution curves. (●) Animal 3 measured by fluoroimmunoassay. (○) Animal 4 measured by fluoroimmunoassay. (×) Animal 4 measured by enzyme immunoassay.

Fluoroimmunoassay. The fluoroimmunoassay was developed by using the fluorescein amine labeled diclofop as a tracer, which was synthesized from diclofop acid and fluorescein amine by the mixed anhydride method. This tracer could be measured fluorometrically with good precision (standard deviation in the tested concentration range 3%) and sensitivity (detection limit 5 ng/mL). The tracer was very stable; the stock solution did not show any decompositon during the period of 1 year. One milligram of the tracer is sufficient for the analysis of more than 30 000 samples so that the specific costs for the tracer are very low.

In the fluoroimmunoassay procedure, polyethylene glycol was used to separate antibody bound from free antigen. The antibody-bound fraction was precipitated almost instantaneously with less than 7% unspecific precipitation of the free tracer.

The fluorescence intensity of the free tracer was measured in the supernatant fluid and increased with increasing concentrations of the unlabeled diclofop-methyl in the samples. The percentage of bound tracer (=B) was calculated from the fluorescence signal. B_0 reflects the value of the bound tracer in the absence of unlabeled diclofop-methyl.

The standard curve shown in Figure 2 was obtained by plotting $B/B_0 \times 100$ vs. the logarithm of the diclofop-methyl concentration. The lower limit of detection—the amount of substance the signal of which is significantly different from the analytical blank—was estimated from the 3s deviation of the B_0 value (antibody binding without the addition of unlabeled diclofop-methyl). It was 9 ng $= 2.6 \times 10^{-11}$ mol/assay tube. For each determination 0.2 mL of the sample was used. Consequently, the lower limit of detection was 45 ng/mL = 45 ppb = 130 nmol/L.

The assay midpoint, i.e., the concentration of diclofop-methyl at $B/B_0 \times 100 = 50\%$, was 32.0 ng/mL $= 9.4 \times 10^{-8}$ mol/L. This means that 32.0 ng of diclofop-methyl can replace 50% of the tracer from its binding to the antibodies. The error of the x values can be calculated from the errors of slope and interception of the regression line. It ranges from 7% in the lower region to 10% in the upper region of the determined concentrations. According to Müller (1980), the average antibody affinity of the used antiserum pool can be estimated from the assay midpoint and the tracer concentration used in the assay procedure. The average antibody affinity constant was calculated as 5.6×10^7 L/mol.

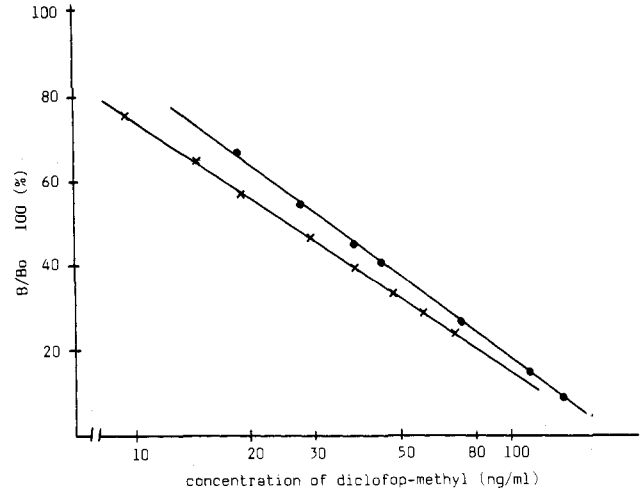


Figure 2. Standard curves for the determination of diclofop-methyl by fluoroimmunoassay (●) and enzyme immunoassay (×).

Enzyme Immunoassay. For the development of the enzyme immunoassay, horseradish peroxidase labeled diclofop was synthesized. The determination of the tracer was optimized by using H_2O_2 and 1,2-phenylenediamine as the substrates. The assay was very sensitive; even 0.06 ng/mL enzyme-labeled tracer could be measured with a standard deviation of 4%. The conjugation step of peroxidase and diclofop was very efficient, because 70% of the enzyme activity of the pure enzyme could be preserved. The performance of the separation step of antibody-bound antigen from free antigen in enzyme immunoassay is more complicated than in fluoroimmunoassay because of the high molecular mass of the enzyme-labeled tracer. In the developed enzyme immunoassay, a combination of the second antibody and polyethylene glycol was used. The addition of polyethylene glycol increased the precipitation velocity of the antibody-bound tracer by the specific second antibody so that the incubation time necessary for the separation step decreased from 12 h to half an hour.

The enzyme activity was measured in the precipitate showing decreasing values on increasing diclofop-methyl concentrations. The percentage of bound tracer (=B) was calculated from the values of the enzyme activity. B_0 signifies the value of tracer bound in the absence of unlabeled diclofop-methyl. The standard curve for the enzyme immunoassay shown in Figure 2 was obtained by plotting $B/B_0 \times 100$ vs. the logarithm of the diclofop-methyl concentration. The lower limit of detection determined as described above in the fluoroimmunoassay procedure was 23 ng/mL = 23 ppb = 67 nmol/L. The errors of the x values estimated as described above ranged from 10% in the lower region of concentration to 14% in the upper region. The average antibody affinity constant in enzyme immunoassay was 4.0×10^7 L/mol.

Antibody Specificity. The antibody specificity was determined by measuring the cross-reactivities of structurally related compounds. The cross-reactivity of a compound A is defined as

$$\% \text{ cross-reactivity} = \frac{\text{moles of diclofop-methyl at the assay midpoint}}{\text{moles of A at the assay midpoint}} \times 100$$

Table II shows compounds with cross-reactivities of more than 10%; Table III shows those with cross-reactivities up to 10%.

Substances that were used in concentrations up to 1000-fold higher than that of diclofop-methyl without showing any influence on the binding of the tracer were listed in Table IV. Their cross-reactivities are less than

Table II. Compounds with Cross-Reactivities of More Than 10%

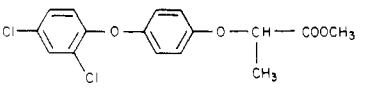
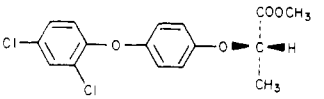
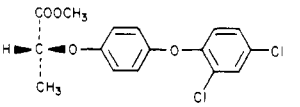
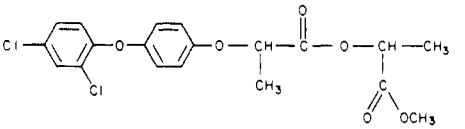
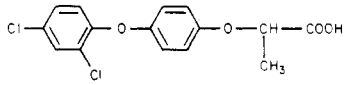
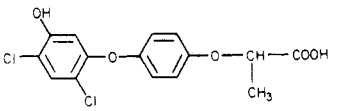
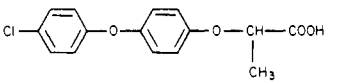
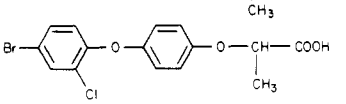
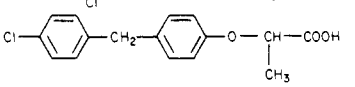
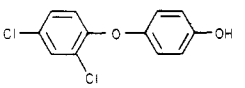
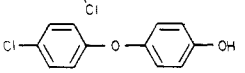
name of compound	formula	cross reactivity, %
methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate		100.0
L-(−) enantiomer of diclofop-methyl		104.8
D-(+) enantiomer of diclofop-methyl		94.2
2-methoxy-1-methyl-2-oxoethyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate		59.4
2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid		33.6

Table III. Compounds with Cross-Reactivities of Less Than 10%

name of compound	formula	cross-reactivity, %
2-[4-(2,4-dichloro-5-hydroxyphenoxy)phenoxy]propanoic acid		2.5
2-[4-(4-chlorophenoxy)phenoxy]propanoic acid		0.7
2-[4-(4-bromo-2-chlorophenoxy)phenoxy]propanoic acid		0.4
2-[4-[(4-chlorophenyl)methyl]phenoxy]propanoic acid		0.5
4-(2,4-dichlorophenoxy)phenol		4.2
4-(4-chlorophenoxy)phenol		0.2

0.1% according to the definition above. The results were obtained with the fluoroimmunoassay and the antiserum pool of animal 3. They did not differ significantly from those measured by enzyme immunoassay or with the antiserum of animal 4. As demonstrated in Table II, the antibodies could not discriminate between the stereoisomers of diclofop-methyl. Within the range of errors, the L-(−) and the D-(+) enantiomer showed the same cross-reactivities, identical with that of the racemic diclofop-methyl. As the racemic form of the acid diclofop was used in the synthesis of the immunogen, a stereoselective antiserum could not be expected.

In addition to diclofop-methyl, two other compounds show considerable cross-reactivities, diclofop acid and the ester 2-methoxy-1-methyl-2-oxoethyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate, elucidating the low specificity of the antibodies toward the carboxylic group of the molecules. As a result of this property of the used antisera, diclofop and other esters of the diclofop series can also be examined by the fluoroimmunoassay and enzyme immunoassay procedures. If a sample contains more than one compound of this group of herbicides, the sum of all can be determined on the base of diclofop acid after an alkaline saponification. Alternatively, each compound could be assayed separately after a simple partition or chromatography step. Figure 3 shows the standard curves for the determination of diclofop by enzyme immunoassay and fluoroimmunoassay. The limits of detection for diclofop

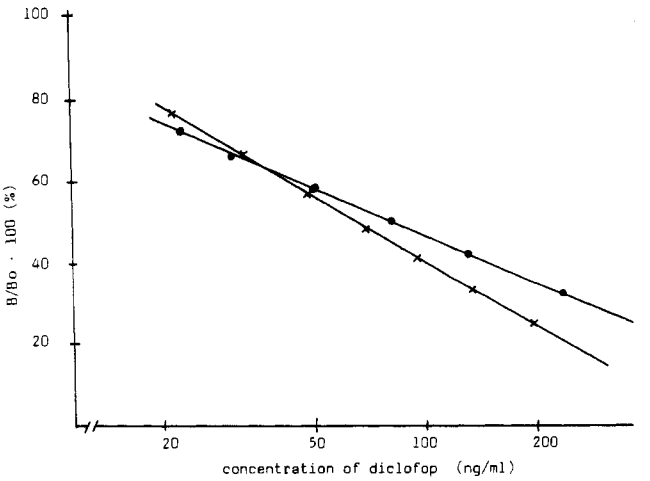
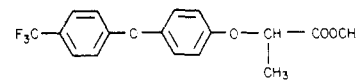
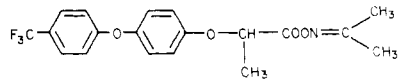
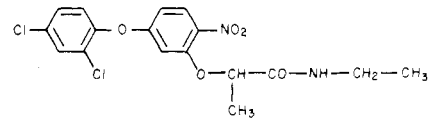
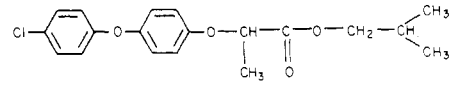
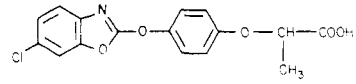
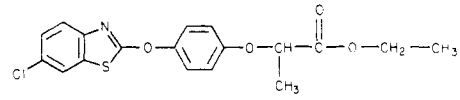
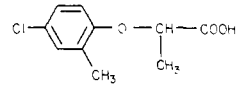
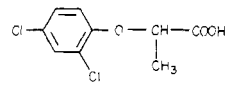
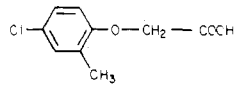
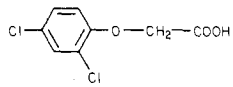
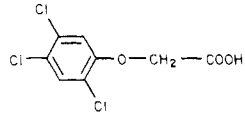
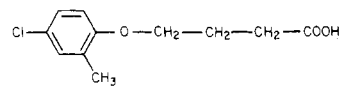
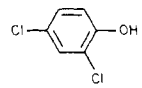
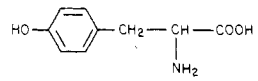
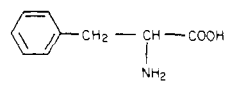
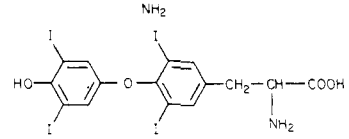
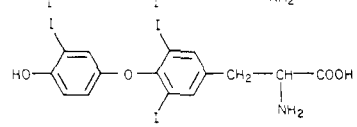


Figure 3. Standard curves for the determination of diclofop by fluoroimmunoassay (●) and enzyme immunoassay (×).

Table IV. Compounds without Measurable Cross-Reactivities

name of compound	formula
2-[4-[4-(trifluoromethyl)phenoxy]phenoxy]propanoic acid	
2-propanone *O*-[1-oxo-2-[4-[4-(trifluoromethyl)phenoxy]phenoxy]propyl]oxime	
N-ethyl-2-[3-(2,4-dichlorophenoxy)-6-nitrophenoxy]propionamide	
2-methylpropyl 2-[4-(4-chlorophenoxy)phenoxy]propionate	
2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]propanoic acid	
ethyl 2-[4-[(6-chloro-2-benzthiazolyl)oxy]phenoxy]propionate	
2-(4-chloro-2-methylphenoxy)propanoic acid (MCPP)	
2-(2,4-dichlorophenoxy)propanoic acid (Dichlorprop)	
(4-chloro-2-methylphenoxy)acetic acid (MCPA)	
(2,4-dichlorophenoxy)acetic acid (2,4-D)	
(2,4,5-trichlorophenoxy)acetic acid (2,4,5-T)	
4-(4-chloro-2-methylphenoxy)butyric acid (MCPB)	
2,4-dichlorophenol	
L-tyrosine	
L-phenylalanine	
thyroxine (T_4)	
triiodothyronine (T_3)	

were 55 ng/mL = 55 ppb = 170 nmol/L in fluoroimmunoassay and 43 ng/mL = 43 ppb = 130 nmol/L in enzyme immunoassay. The errors of the x values were in the same range as for the determination of diclofop-methyl. Tables III and IV show that the cross-reactivities decreased considerably if there were structural differences in the aromatic moiety of the molecules. From the percentages of the measured cross-reactivities, it can be deduced that the specificity of the antibodies is primarily directed against the (2,4-dichlorophenoxy)phenoxy part of the herbicide. This means that most of the antibody specificity is directed against that part of the hapten that was as distant as possible from the functional group used for the coupling to the protein carrier.

Compounds that do not possess the 4-(2,4-dichlorophenoxy)phenoxy structure did not show any cross-reactivity. Therefore, even the closely related herbicides dichlorprop or 2,4-D do not interfere with the determi-

Table V. Recoveries of Diclofop-methyl and Diclofop in Fortified Extracts of Natural Samples Determined by Enzyme Immunoassay (EIA) and Fluoroimmunoassay (FIA)[a]

method	compound	concn in the fortified extract, ng/mL	recovery, %	no. of determinations
FIA	diclofop-methyl	62.5	99.1	6
		75.0	99.8	4
		100.0	97.1	4
		125.0	97.1	3
		150.0	99.2	3
		250.0	96.5	4
			97 ± 5.7	24
FIA	diclofop	100.0	97.4	4
		250.0	96.3	5
		400.0	97.4	3
		500.0	97.4	6
			97.1 ± 7.9	18
EIA	diclofop-methyl	37.5	108.0	1
		50.0	93.0	1
		75.0	94.8	1
		100.0	109.5	1
		125.0	102.2	2
		150.0	95.7	2
		200.0	101.3	2
		250.0	103.8	2
			100.9 ± 7.6	12
EIA	diclofop	75.0	105.7	2
		100.0	91.8	2
		125.0	105.2	1
		150.0	94.3	1
		200.0	105.2	2
		250.0	102.4	2
		350.0	101.7	2
			99.3 ± 7.7	12

[a] Each determination was carried out in duplicate. If more than one determination was performed, the value of recovery gives the mean value of all the determinations.

Table VI. Detection Limits of Diclofop-methyl and Diclofop in Natural Samples

sample	diclofop-methyl in FIA, ppm	diclofop in FIA, ppm	diclofop-methyl in EIA, ppm	diclofop in EIA, ppm
soil	0.045	0.055	0.023	0.043
milk	0.450	0.550	0.230	0.430
urine	0.225	0.275	0.115	0.215
normal rabbit serum	0.180	0.220	1.150	2.150
sugar beets	0.225	0.275	1.150	2.150
soybeans	0.225	0.275	0.115	0.215
grains of wheat	0.450	0.550	0.230	0.430
wheat shoots	9.000	11.000	4.600	8.600
buffer	0.045	0.055	0.023	0.043

nation of diclofop-methyl or diclofop. Thus, the produced antibodies are highly specific for the diclofop structure.

Processing of Biological Samples. For the application of the immunological procedures, various biological samples were extracted with an organic solvent. The organic solution was evaporated and the residue taken up in assay buffer. No further cleanup was used. The aqueous phase was diluted to a degree that no disturbance of the fluorescence measurement or the determination of the enzyme activity occurred.

To these final solutions, known amounts of diclofop-methyl or diclofop were added. The resulting concentrations in these solutions ranged from 37.5 to 250 ng/mL for diclofop-methyl and from 75.0 to 500.0 ng/mL for diclofop. The recoveries were determined either by fluoroimmunoassay or by enzyme immunoassay. The results of these experiments are shown in Table V. The presence of compounds originating from the biological samples diluted as described under Materials and Methods did not interfere with the determination of diclofop-methyl or diclofop by fluoroimmunoassay and enzyme immunoassay. The limits of detection of the compounds in the various biological samples are given in Table VI. The results indicate that the immunological procedures described in this paper allow the determination of diclofop-methyl and diclofop in many different biological samples without the need of a time-consuming cleanup. Therefore, fluoroimmunoassay and enzyme immunoassay are suitable for the routine analysis of the compounds.

Reference Methods. In order to control for the values obtained by the immunological procedures, gas chromatography and liquid scintillation counting were used as reference methods. Samples that contained diclofop-methyl were extracted with hexane as described under Materials and Methods. Part of this hexane extract was injected into the gas chromatograph. The fractions of the biological samples showed interfering substances but did not influence the measurement of diclofop-methyl since the retention time of the methyl ester was 14.0 min, and the impurities were eluted earlier. The limit of detection of the gas chromatographic determination was 50 ng/mL = 50 ppb. The values obtained by gas chromatography correlated well with those of the fluoroimmunoassay (r = 0.996, 12 samples) and with those of the enzyme immunoassay (r = 0.99, 8 samples).

Samples that contained ^{14}C-labeled diclofop-methyl or ^{14}C-labeled diclofop were examined by liquid scintillation counting of radioactivity as described under Materials and

Methods. Interferences of compounds originating from biological material were controlled by adding known amounts of the radioactive ester and determining the counting efficiency. If a maximum standard deviation of 10% is accepted, the limit of detection of the scintillation counting of ^{14}C-labeled diclofop-methyl or diclofop was 20 ng/mL = 20 ppb. The values obtained by liquid scintillation counting correlated well with those measured by fluoroimmunoassay (r = 0.991, 19 samples) and enzyme immunoassay (r = 0.99, 12 samples).

Conclusions. The fluoroimmunoassay and enzyme immunoassay procedures described in this paper allow the determination of the herbicides diclofop-methyl and diclofop in various biological samples with good sensitivity and high specificity. The results indicate that immunological methods are suitable for the routine analysis of pesticides. Because of the specificity of the antigen–antibody reaction, there is no need for a time-consuming cleanup of the samples. Enzyme immunoassay and fluoroimmunoassay are suitable for the quantification of herbicides at the nanogram level, but the enzyme immunoassay requires more time and is more expensive than the fluoroimmunoassay because of the time-consuming procedures for separating antibody bound from free antigen and because of the additional steps for determining the enzyme activity. The fluoroimmunoassay allows the analysis of about 60 samples per day with a minimum of reagents and equipment. The sensitivity of the immunoassay procedures probably can be improved if a heterogeneous immunoassay system is used. This means that, for example, instead of diclofop the 5-hydroxy compound [2-[4-(2,4-dichloro-5-hydroxyphenoxy)phenoxy]propanoic acid] is used as hapten in the immunogen and coupling is performed via the 5-hydroxy group. However, this would probably change the specificity of the antibodies. It is well-known that the specifity of an antiserum is highest toward those groups of the antigen that are most distant from the coupling site in the immunogen.

ACKNOWLEDGMENT

We thank Dr. S. Baudner from the Behringwerke AG in Marburg, West Germany, for the performance of the immunization.

Registry No. Diclofop-methyl, 51338-27-3; L-(−)-diclofop-methyl, 75021-72-6; D-(+)-diclofop-methyl, 71283-65-3; 2-methoxy-1-methyl-2-oxoethyl 2-[4-(2,4-dichlorophenoxy)phenoxy]-propionate, 66497-51-6; 2-[4-(2,4-dichlorophenoxy)phenoxy]-propanoic acid, 40843-25-2; 2-[4-(2,4-dichloro-5-hydroxyphenoxy)phenoxy]propanoic acid, 61955-11-1; 2-[4-(4-chlorophenoxy)phenoxy]propanoic acid, 26129-32-8; 2-[4-(4-bromo-2-chlorophenoxy)phenoxy]propanoic acid, 64196-91-4; 2-[4-[(4-chlorophenyl)methyl]phenoxy]propanoic acid, 57081-31-9; 4-(2,4-dichlorophenoxy)phenol, 40843-73-0; 4-(4-chlorophenoxy)-phenol, 21567-18-0.

LITERATURE CITED

Ercegovich, C. D.; Vallejo, R. P.; Gettig, R. R.; Woods, L.; Bogus, E. R.; Mumma, R. D. *J. Agric. Food Chem.* **1981**, *29*, 559–563.
Fatori, D.; Hunter, W. M. *Clin. Chim. Acta* **1980**, *100*, 81–90.
Hunter, K. W.; Lenz, D. E. *Life Sci.* **1982**, *30*, 355–361.
Langone, J. J.; Van Vunakis, H. *Res. Commun. Chem. Pathol. Pharmacol.* **1975**, *10*, 163–171.
Levitt, T. *Lancet* **1977**, *8033*, 358.
Levitt, T. *Proc. Anal. Div. Chem. Soc.* **1979**, *16*, 72–76.
Lukens, H. R.; Williams, C. B.; Levinson, S. A.; Dandliker, W. B.; Murayama, D.; Baron, R. L. *Environ. Sci. Technol.* **1977**, *11*, 292–297.
Müller, R. *J. Immunol. Methods* **1980**, *34*, 345–352.
Nestler, H. J. In "Chemie der Pflanzenschutz- und Schädlingsbekämpfungsmittel, Band 8"; Springer-Verlag: Berlin and Heidelberg, West Germany, 1982; pp 1–26.
Newsome, W. H.; Shields, J. B. *J. Agric. Food Chem.* **1981**, *29*, 220–222.
Rinder, D. F.; Fleeker, J. R. *Bull. Environ. Contam. Toxicol.* **1981**, *26*, 375–380.
Tessari, J. D.; Savage, E. P. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 736–741.
Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. *J. Agric. Food Chem.* **1982**, *30*, 572–580.
Wie, S. I.; Hammock, B. D. *J. Agric. Food Chem.* **1982**, *30*, 949–957.
Wie, S. I.; Sylwester, A. P.; Wing, K. D.; Hammock, B. D. *J. Agric. Food Chem.* **1982**, *30*, 943–948.
Wing, K. D.; Hammock, B. D. *Experientia* **1979**, *35*, 1619–1620.
Wing, K. D.; Hammock, B. D.; Wustner, D. A. *J. Agric. Food Chem.* **1978**, *26*, 1328–1333.

Received for review October 11, 1983. Accepted February 22, 1984.