Development of an Enzyme-Linked Immunosorbent Assay for the Detection of the Herbicide Clomazone

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An enzyme-linked immunosorbent assay (ELISA) for clomazone, the active ingredient in the soybean herbicide Command, was developed. The ELISA system proved to be a fast, sensitive, and accurate method of clomazone detection. The assay was not affected by soil type; however, soil extracts did influence detection sensitivity. The assay was specific for clomazone and did not detect the soybean herbicides metribuzin, metolachlor, or trifluralin.

INTRODUCTION

Immunoassay detection technology has been used extensively in medicine and pharmacology (Erlanger et al., 1959; Marks, 1974; Spector, 1971). In agriculture, immunoassay has been found to be an accurate and efficient method for pesticide detection (Hammock and Mumma, 1980; Vanderlaan et al., 1988). Immunoassay detection systems have been developed for the herbicides atrazine (Bushway et al., 1988; Huber, 1985), chlorsulfuron (Kelly et al., 1985), diclofop-methyl (Schwalbe et al., 1984), molinate (Gee et al., 1988), paraquat (Niewola et al., 1983, 1985), terbutryn (Huber and Hock, 1985), and 2,4-D and 2,4,5-T (Rinder and Fleeker, 1981).

Clomazone, the active ingredient in the herbicide Command, is applied as a preplant-incorporated treatment at rates of 0.56–1.12 kg/ha for control of both grass and broadleaf weeds in soybean (FMC Corp., 1987). Residual levels of clomazone have been shown to be injurious to susceptible rotational crops such as corn, wheat, and alfalfa (Gunsolus et al., 1986). Clomazone levels of 0.20 and 0.10 ppm have injured corn and wheat, respectively, on a Drummer silty clay loam soil in Illinois (Curran et al., 1987). In field studies, the half-life of clomazone ranged from 15 to 45 days depending on soil type (FMC Corp., 1987).

Present methods of clomazone detection include gas chromatography, high-performance liquid chromatography, and wheat shoot bioassay (Loux et al., 1989). These methods are effective but require extensive sample cleanup procedures and expensive equipment and are time-consuming. Enzyme-linked immunosorbent assay (ELISA) offers an alternative for clomazone detection in soil. Immunoassay technology is based on the principle that animal immune systems can produce specific antibodies to foreign substances (Engvall and Perlman, 1971). A herbicide-specific antibody competes for free herbicide and herbicide–protein conjugate adsorbed to polystyrene plates. The amount of herbicide-specific antibody bound to the polystyrene plate, via the herbicide–protein conjugate, is determined by using an enzyme-labeled anti-immunoglobulin and relevant substrate.

This study describes the development of an ELISA that is both specific and sensitive to clomazone and that can be used to detect clomazone in soil extracts.

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EXPERIMENTAL PROCEDURES

Caution. Precaution is advised during the nitration and hydrogenation processes that are mentioned in this paper: avoid contact, wear rubber gloves and a labcoat, avoid inhalation, and work in a fume hood.

Nitration of Clomazone. Procedures developed by Kaslow and Buchner (1958) were modified for the nitration of clomazone. Concentrated nitric acid, 5.2 mL (0.083 mol), in 20 mL of concentrated sulfuric acid was added dropwise to a stirred solution of 20 g (0.083 mol) of clomazone [2-[(2-chlorophenyl)-methyl]-4,4-dimetyl-3-isoxazolidinone] dissolved in 65 mL of concentrated sulfuric acid. The addition was carried out over a 7-h period while a reaction temperature of −10 °C was maintained. The nitration mixture was warmed to 0 °C and then poured onto 400 mL of ice and water. Concentrated ammonium hydroxide was added to the mixture, resulting in precipitation of the nitrated product at a slightly acid pH as determined by broad-range pH paper. The precipitate was removed by filtration, washed with cold dilute nitric acid, and air-dried. The yield of nitrated clomazone was 17 g. Addition of the nitro group to clomazone was indicated by fast atom bombardment mass spectrometry (FABMS) m/z (relative intensity) 287 and 285 ($M^+ + H$, 53 and 18), 242 and 240 ($M^+ - NO - CH_3$, 23 and 7), in agreement with the FABMS obtained from p-nitroclomazone standard provided by FMC Corp.

Hydrogenation of Nitroclomazone. To a solution of 0.50 g of nitroclomazone in 155 mL of ethanol was added 0.50 mg of platinum oxide catalyst. The mixture was shaken in a 500-mL thick-walled bottle on a Parr hydrogenator under hydrogen at 45 psi. After 60 min, the pressure dropped 2 psi. The resulting clear, yellow solution was filtered through diatomaceous earth under suction to remove the catalyst, and the filtrate was evaporated to dryness in vacuo at temperatures below 30 °C. Reduction of NO_2 to NH_2 was indicated by FABMS m/z (relative intensity) 257 and 255 ($M^+ + H$, 85 and 30), in agreement with the FABMS obtained from a p-aminoclomazone standard provided by FMC Corp.

Conjugate Preparation. Aminoclomazone, 2 g (7.8 mmol), was added to hydrochloric acid, 100 mL (0.1 N) and cooled to 4 °C. To this solution (0.1 N) was added $NaNO_2$ dropwise until a positive starch–iodine test was obtained. The reaction mixture was added to 4 g of bovine serum albumin (BSA, RIA grade, Sigma) that was dissolved in 100 mL of borate buffer, pH 9.0. The mixture was stirred for 2 h at 4 °C, while a pH of 9.0 was maintained by addition of 0.1 N NaOH. The bright orange reaction mixture was dialyzed for 3 days against a saline buffer solution (0.1 M, pH 7.2). The same procedure was used to conjugate bovine γ-globulin (BGG, Sigma) with aminoclomazone for use as a coating antigen.

Antibody Production. Two female New Zealand white rabbits were used to obtain antibodies against the BSA–clomazone (BSA–C) conjugate. The rabbits were subcutaneously

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injected on three to four sites of the lower back with 1.5 mg of conjugate in 1 mL of Freund's complete adjuvant. Booster injections were given 2 weeks after initial injection and at monthly intervals thereafter, using 0.15 mg of conjugate in 1 mL of Freund's incomplete adjuvant. Blood was collected 6 weeks after initial injection and at 2-week intervals thereafter. The cleared serum was stored at −70 °C in 1-mL samples. Both rabbits produced useful antibodies 6 weeks after immunization. Pooled antibodies from several harvest dats demonstrated comparable activity in the ELISA screen. To standardize the assay, serum collected from a single rabbit 5 months after the initial injection was used in all studies.

ELISA Procedure. The coating antigen, BGG–clomazone (BGG–C) at 10 ng/mL, in Na_2CO_3 (0.1 mM, pH 9.6) coating buffer was added to 96-well polystyrene plates (Costar EIA No. 3590) at 0.2 mL/well. The coating antigen was allowed to bind to the plate overnight at 4 °C. The following day, the plates were washed six times with phosphate-buffered saline (PBS, pH 7.2, Sigma) plus 0.05% Tween 20 (PBS–T, Monsanto Corp.). Antiserum diluted 1:1000 with PBS–T was added to the wells at 0.2 mL/well. After a 2-h incubation period at 37 °C, the wells were washed six times with PBS–T to remove unreacted antisera. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) (1:1000 in PBS–T) was added at 0.1 mL/well and allowed to incubate for 2 h at 37 °C. The plate was again washed six times with PBS–T. *p*–Nitrophenyl phosphate substrate (Sigma) in 10% diethanolamine buffer was added at 0.2 mL/well, and the plate was incubated at 37 °C. Absorbance, in each well, was determined 20–30 min after incubation on a Bio Tek Instruments EL 309 plate reader at 405 nm.

Inhibition Studies. Similar procedures were used to determine the detection parameters of the assay and to quantify clomazone in soil extracts. Free herbicide was dissolved in PBS–T and incubated with an equal volume of clomazone-specific antisera in a glass tube for 1 h at 20 °C. Following incubation, 0.2 mL/well of the herbicide/antisera mixture was added to the wells of polystyrene plates containing BGG–C coating antigen for 1 h at 20 °C. The amount of previously unbound antisera was determined by using standard solid-phase ELISA procedures. Color development, attributed to *p*-nitrophenol production, is directly proportional to the amount of unbound antibody and indirectly proportional to the original concentration of free herbicide.

Soil Extraction Procedure. Varying extraction procedures were used to determine their effect on immunoassay detection. A modification of the procedure developed by Loux et al. (1989) was used to extract soil. Extraction was performed by shaking 50 g of soil with 100 mL of acetonitrile for 1 h, followed by centrifugation for 10 min at 1000*g* (one-step extraction). The procedure was repeated one additional time for the two-step extract and a third time for the complete, three-step extraction. The acetonitrile fractions were combined and filtered. Following filtration, the extracts were evaporated to dryness at 30 °C on a rotary evaporator, and the residue was resuspended in 2 mL of PBS–T. Buffer extraction of soil was performed by shaking 50 g of soil with 50 mL of PBS–T for 18 h followed by filtration.

All four extracts (one-step, two-step, three-step, buffer) were spiked with either 0.01, 0.1, 1, or 10 ppm of clomazone for use in the competition assay described above.

RESULTS AND DISCUSSION

The chemical structure of clomazone was modified to prepare the herbicide for protein conjugation, according to the reaction sequence shown in Figure 1. The product of the nitration reaction is believed to be a mixture of isomers with substitution occurring on the aromatic ring ortho and para to the aliphatic substituent and meta to the chloro group (Olah et al., 1962). Under these conditions (nitric acid in sulfuric acid at −10 °C), no nitration in the aliphatic portion of the molecule is anticipated. These expectations are supported by fast atom bombardment mass spectral analysis, which not only yields $M^+ + H$ ions at 287 and 285, identical with that given by a *p*-nitroclomazone standard of independent origin, but also gives significant peaks at 242 and 240, corresponding to loss of CH_3 α to the keto group, combined with loss of NO, which is diagonistic for structures containing aromatic NO_2 (Silverstein and Bassler, 1976).

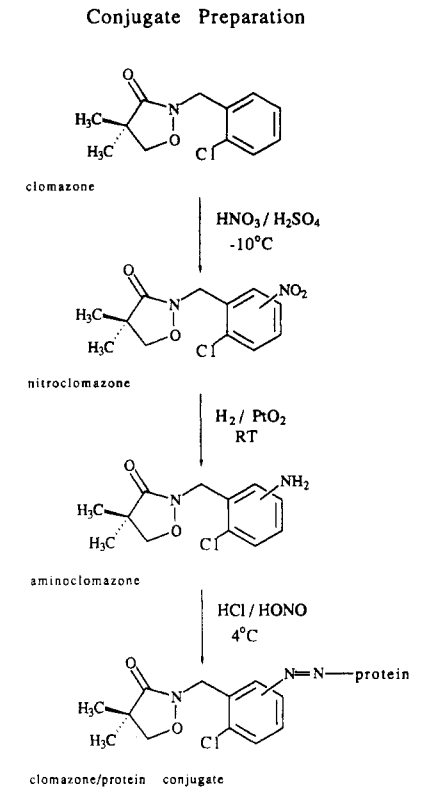

Figure 1. Reaction scheme for the synthesis of clomazone/protein conjugates.

Reduction of the nitro derivative by catalytic hydrogenation is believed to give a mixture of the corresponding aminoclomazone. Under these conditions (45 psi, 1 h, platinum oxide), no reduction of the keto group, loss of Cl, or reduction of the aromatic or heterocyclic rings is anticipated (Rylander, 1967). The expectations are also supported by FABMS analysis, which shows $M^+ + H$ ions at 257 and 255 (in a ratio of 1:3, indicating the presence of Cl) identical with that given by a *p*-aminoclomazone standard of independent orgin. A large $M^+ - 18$ peak, which would be anticipated from an aliphatic amine, was not observed (Silverstein and Bassler, 1976).

The above assignments of structure, while strongly indicative, are nonetheless tentative, but further purification and characterization was not deemed necessary, since attachment of the amino derivatives to protein would be expected to obscure the aromatic end of the molecule from antibody. Rather, recognition is probably directed toward the unhindered heterocyclic portion of the molecule, whose structure (and hence recognition) is independent of the precise location of attachment of the amino (or nitro) group within the aromatic ring.

A dose–response curve of rabbit antiserum for dilutions ranging from 1 in 10 to 1 in 1 million was prepared by using standard ELISA procedures (Figure 2). Reactivity was demonstrated over the entire range of antisera dilutions tested and demonstrates the antibody binding capacity to the clomazone coating antigen (BGG–C).

When free clomazone was added to the antisera, ELISA detection with the clomazone competition reaction ranged from 0.5 to 500 pbb (Figure 3). The detection range compares favorably with other methods of clomazone

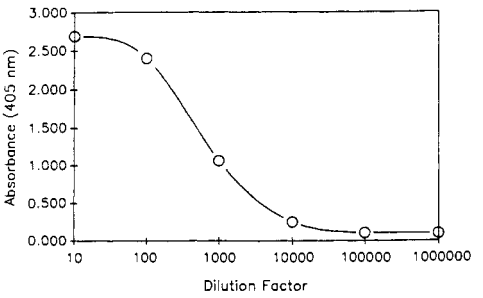

Figure 2. Dose–response curve for clomazone antiserum. (Data are means of two separate experiments run in triplicate.)

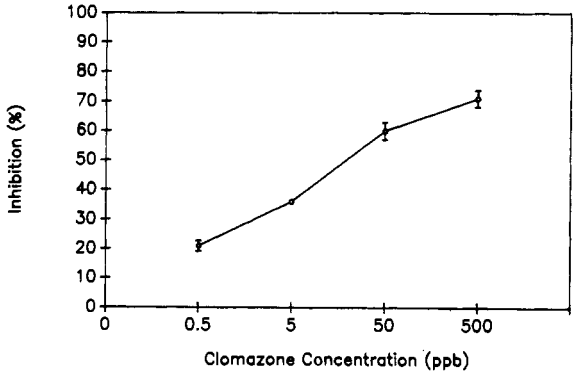

Figure 3. Range of infused clomazone detection in phosphate buffer by ELISA. (Means and standard errors are calculated from two separate experiments run in triplicate.)

detection. The wheat shoot bioassay has a detection limit near 50 ppb (Loux et al., 1989). Acetonitrile extraction and HPLC were used to detect clomazone in soils treated with 1–5 ppb of the herbicide (Curran et al., 1987; Loux et al., 1989). Verification of clomazone detection with ELISA was provided by gas chromatography which had similar detection efficiencies on several types of clomazone-treated soil (Koppatschek et al., 1990).

The soybean herbicides clomazone, metolachlor, metribuzin, and trifluralin, as well as clomazone conjugate derivatives, were used for I_{50} determinations (Figure 4). The amount of herbicide needed to produce 50% inhibition relative to the assay without free herbicide is referred to as the I_{50} value. Free clomazone produced an I_{50} value of 12 ng/mL. Vanderlaan et al. (1988) suggested that a pesticide immunoassay with high sensitivity needed I_{50} values approaching 1 ng/mL. The two conjugation derivatives, nitroclomazone and aminoclomazone, had I_{50} values of 6 and 19.5 ng/mL, respectively. The minor differences in I_{50} values between the parent herbicide and the conjugation derivatives reflect similarities in chemical structure. Metribuzin, metolachlor, and trifluralin all had I_{50} values greater than 1000 ng/mL. These herbicides are structurally unrelated to clomazone, but are often applied in combination with clomazone to obtain broad spectrum weed control. The data suggest that clomazone in soil samples could be analyzed by using this immunoassay without interference from these herbicides. The immunoassay offers an advantage over the bioassay systems, which can give false positives if more than one herbicide is present in the soil (Curran et al., 1987).

Plainfield sand (Typic Udipsamments) and a Drummer silty clay loam soil (Typic Haplaquol) two soils with diverse properties, were extracted by using methods for HPLC sample preparation (Table I). The extracts were spiked with clomazone to obtain concentrations of 0.01, 0.1, 1, and 10 ppm. As the concentration of clomazone increased, inhibitory activity increased. Although no difference in clomazone detection was observed between the two soils,

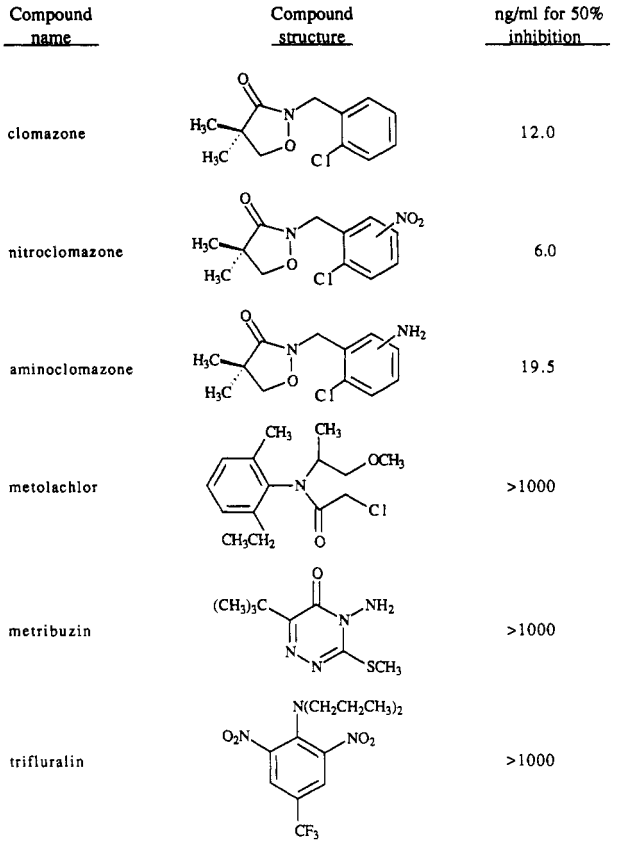

Figure 4. Structures and activities of compounds assayed for reactivity to clomazone antisera by ELISA. I_{50} values represent the herbicide concentration that inhibits 50% of the zero analyte ELISA absorbance value.

Table I. Soil Properties of Drummer Silty Clay Loam and Plainfield Sand

soil type	pH	sand, %	silt, %	clay, %	organic content, %
Drummer silty clay loam	5.7	9	57	34	5.8
Plainfield sand	6.2	98	1	1	0.4

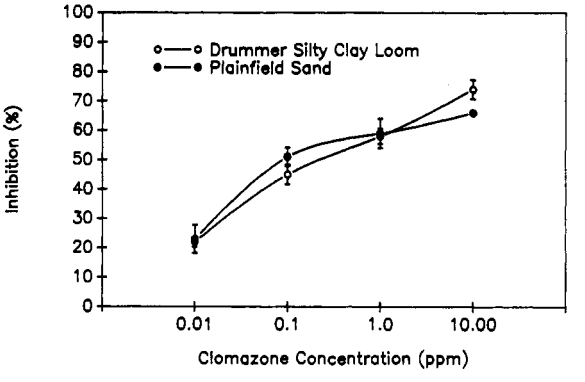

Figure 5. Influence of soil type on competitive inhibition with infused clomazone. Clomazone was infused into soil extracts. (Means and standard errors are calculated from two separate experiments run in triplicate.)

soil extracts did contribute to decreased inhibitory activity (Figure 5). The decrease in assay sensitivity from soil extracts was similar for all clomazone concentrations in both soils. The decrease in assay sensitivity may be attributable to soil components, remaining after extraction, binding with clomazone and limiting the amount of clomazone available for bonding with antisera. Although the procedure is somewhat less effective when soil extracts are included, the immunoassay can be used for clomazone detection on a wide range of soils while maintaining similar detection efficiencies. Kelly et al. (1985) found that soil

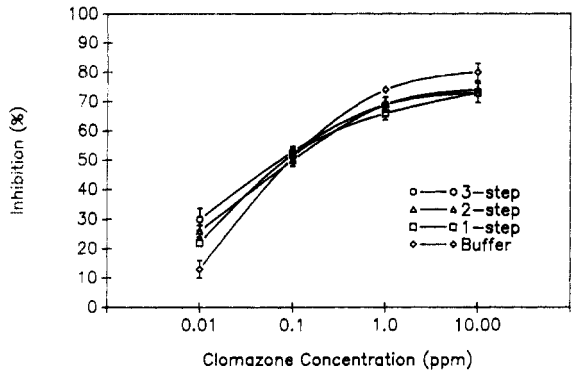

Figure 6. Effect of extraction type and the extent of extraction on competitive inhibition with infused clomazone. Acetonitrile extraction was performed either one, two, or three times. A phosphate buffer extract was also performed. (Means and standard errors are calculated from two separate experiments run in triplicate.)

type and extracts have an influence on chlorsulfuron detection with ELISA.

Drummer silty clay loam soil was extracted by using several levels of acetonitrile extraction and a phosphate buffer extraction. Varying the level of acetonitrile extraction had no influence on immunoassay detection of clomazone (Figure 6). Clomazone detection was reduced in phosphate buffer extracts at the 0.01 ppm concentration of clomazone compared to the three- two-, and one-step acetonitrile extracts (Figure 6). This result suggests that the soil components remaining after the phosphate buffer extraction may interfere with ELISA detection of low levels of clomazone. However, a phosphate buffer extraction of soil would be an attractive method for quickly determining the presence of clomazone in a soil sample. Reducing the number of acetonitrile extraction steps needed for analysis would be both time and cost effective.

Immunoassay has potential use for detecting herbicide residues in soil. The system offers a fast, accurate, and possibly inexpensive method of herbicide detection. These advantages make immunoassay attractive for use in herbicide carryover detection on midwestern soils. However, immunoassay technology is presently not sufficiently simplified or widely available for use by agriculturalists in the field. Pesticide scientists have proven many times that immunoassay is a viable method for pesticide detection. Making the technology available for field use is the next step in herbicide immunoassay development.

ABBREVIATIONS USED

BSA, bovine serum albumin; BGG, bovine γ-globulin; BSA–C, bovine serum albumin conjugated to clomazone; BGG–C, bovine γ-globulin conjugated to clomazone; ELISA, enzyme-linked immunosorbent assay; FABMS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; I_{50}, the concentration inhibiting 50% of the zero analyte ELISA absorbance value; PBS–T, phosphate-buffered saline and Tween 20.

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