Clomazone Measurement by Enzyme-Linked Immunosorbent Assay

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An indirect, competitive, enzyme-linked immunosorbent assay (ELISA) was developed for the measurement, in soil, of clomazone [FMC 57020, 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazo-lidinone], the active ingredient of Command herbicide. The *p*-aminophenyl analogue of clomazone was used to prepare a bovine serum albumin conjugate for immunization of rabbits. With soil, the sensitivity limit is about 10 ppb and the detection limit is about 5 ppb. The detection range in soil extends from 10 ppb to 1.25 ppm. The method described here uses simple aqueous extraction of soil and permits a throughput of several hundred samples per week when a computer-controlled plate reader is used. The correlation coefficient of this method compared to a GLC method by linear regression is 0.98. A correlation between the amount of clomazone herbicide residue detected in soil by ELISA and the level of plant injury it caused in bioassay is demonstrated. Several analogues of clomazone, substituted in the para position of the phenyl ring, showed cross-reactivity with the antibody. None of the cross-reacting analogues are metabolites of clomazone. In general, substitutions on the isoxazolidinone ring resulted in large decreases in cross-reactivity.

Immunoassays are widely used in clinical chemistry laboratories for medical diagnostic purposes and have been adapted to the quantitative analysis of proteins, hormones, pathogens, and xenobiotics in biological fluids (Wisdom, 1976). In recent years, immunoassays for pesticides have become accepted as methods that complement traditional analytical procedures. Hammock and Mumma (1980) and Mumma and Brady (1987) have reviewed the use of immunoassays for pesticide analysis. Desirable features of immunoassays include high throughput, convenient sample preparation, high specificity, high sensitivity, economy, and the feasibility of automation. In general, the small amounts of waste generated by enzyme immunoassays are easily disposed. Of the variety of enzyme immunoassays, the enzyme-linked immunosorbent assay (ELISA) is, perhaps, the most convenient to set up and use (Engvall, 1980) and has been applied to the herbicides chlorsulfuron (Kelly et al., 1985), paraquat (Van Emon et al., 1986), diclofop-methyl (Schwalbe et al., 1984), and atrazine (Huber, 1985) and the insecticides diflubenzuron (Wie and Hammock, 1982), parathion (Vallejo et al., 1982), and paraoxon (Hunter and Lenz, 1982).

Command herbicide is a preemergence herbicide used for broad-spectrum weed control in soybeans (Chang, 1983). Clomazone [FMC 57020, 2-[(2-chlorophenyl)-methyl]-4,4-dimethyl-3-isoxazolidinone], the active ingredient in Command herbicide, inhibits pigment synthesis and causes a characteristic bleaching in affected plants (Duke and Kenyon, 1986). An ELISA for clomazone has been developed and is described here. A simple, one-step aqueous extraction of soil is followed by quantitation using an indirect, competitive ELISA. We present data and discuss the utility of this new immunoassay for studying the persistence of clomazone residues in soil and the effect of residues on sensitive plant species, especially the potential rotational crop wheat. Additional results and use of this assay have been presented and discussed elsewhere (Dargar et al., 1989).

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MATERIALS AND METHODS

Reagents. Clomazone and its analogues were synthesized in our laboratories. Bovine serum albumin (BSA, fraction V), rabbit serum albumin (RSA), gelatin from cold water fish skin (45% solution), dicyclohexylammonium, *p*-nitrophenyl phosphate (PNPP), and goat anti-rabbit IgG–alkaline phosphatase (IgG–AP) conjugate were purchased from Sigma Chemical Co. HPLC-grade *n*-hexane was obtained from J. T. Baker Chemical Co. All other chemicals were of the highest commercial grade available. The 4EC and 6EC formulations are sold as Command herbicide by FMC Corp. The microencapsulated (MEC) formulation of clomazone was prepared by Pennwalt Corp. Technical grade clomazone (solubility in water 1.1 g/L, vapor pressure 19.2 mPa at 25 °C) was used as a standard in the assays described.

Instruments. The ELISA analyses were carried out in polyvinyl microtiter plates with 96 U bottom wells (Dynatech). The absorbance readings were made by using a plate reader (Dynatech MR 650) at 410 nm. The data were collected and analyzed by linear regression using the Immunosoft program (Dynatech) on an Apple IIC computer. Gas chromatography was performed on a Hewlett-Packard Model 5890A equipped with NP detector, HP 7376A autosampler, and a DB-5 capillary column (0.530 mm × 15 m fused silica, J&W Scientific, Inc.). The temperatures used for analysis were 190 (column), 250 (injection), and 300 °C (detector). Quantitative measurements based on peak area integration were performed by an HP 3292A integrator.

Preparation of Protein Conjugates. All the steps of immunogen preparation were performed at 0 °C and with stirring. *p*-Aminophenyl analogue of clomazone (Chang, 1985) (127.2 mg, 0.5 mmol) was dissolved in 15 mL of 0.167 M hydrochloric acid. To this solution was added sodium nitrite (0.052 M) dropwise until a positive starch iodide test was obtained (Tijssen, 1985). After 30 min of additional stirring, 5.0 mL of the diazotization mixture was slowly added to BSA (25 mL, 5 mg/mL in 0.1 M sodium borate, pH 9.0); the pH was maintained by addition of 5.0 M NaOH. The mixture was stirred for 2 h and then dialyzed against 10 mM sodium phosphate (pH 7.4) containing 0.02% sodium azide. The molar substitution ratio of hapten to BSA was calculated to be 19.2 by using the equations of Fenton and Singer (1971), assuming no loss of protein during dialysis. A similar RSA conjugate with a substitution ratio of 2.4 (coating antigen) was prepared according to the same procedure except the concentration of RSA used in the coupling step was 20 mg/mL.

Antiserum. The immunization and bleeding procedures were performed by Pel-Freeze Biologicals, Rogers, AR. Three New Zealand white rabbits were administered 1 mg of BSA conjugate

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in several intradermal sites (1:1 emulsification in Freund's complete adjuvant) at 0, 2, and 4 weeks. Boosters (1 mg intradermal and 0.5 mg intravenous) were given at 4–6-week intervals, and the animals were bled 10–14 days after each booster injection. To standardize the assay, antiserum collected from rabbit 2, 6 months after the initial immunization, was used.

Selection of ELISA Conditions. The RSA conjugate coat concentration that gave maximum inhibition with 1 ppb of clomazone was chosen as optimal coat concentration. The greatest inhibition of binding of anticlomazone antibody to the solid-phase antigen was obtained as 0.3 μg/mL at various antiserum dilutions (data not shown). Antiserum concentration selected at 1:40 000 gave a desired absorbance of 1.0–1.2 in the absence of free clomazone and was found to be low enough to become the limiting factor in the assay giving an easily measurable response.

ELISA Procedure. All incubations were carried out at ambient temperature (22–23 °C) in a chamber containing moistened paper towels. Clomazone standards were prepared by diluting a stock solution of clomazone 1 mg/mL in Tris-buffered saline (TBS) (20 mM Tris-HCl, 146 mM NaCl, pH 7.4) with deionized water to final concentrations of 1–250 ng/mL.

(1) All microtiter wells except three were incubated overnight with 200 μL of TBS containing 0.3 μg/mL RSA conjugate in TBS. The remaining three wells (blank) contained 200 μL of TBS. The wells were washed; washing consisted of filling and emptying the wells three times with washing buffer (WB, TBS containing 0.05% Tween 20) by using a vacuum aspirator.

(2) The wells were filled with TBS containing 1.0% gelatin and 0.05% sodium azide for 1–1.5 h to block unoccupied sites and then washed with WB as above.

(3) Samples, analogues or standards (100 μL), followed by 100 μL of antiserum solution (prepared by diluting anti-clomazone antiserum 1/40 000 in 2× WB containing 0.1% gelatin) were added to the wells. Following a 1-h incubation, the wells were washed as above.

(4) IgG–AP conjugate (200 μL) diluted 1/1000 in WB containing 0.1% gelatin was added, and after 2 h the wells were washed.

(5) Substrate (215 mL; 1 mg/mL PNPP in 1 M diethanolamine, 1 mM MgCl₂; pH 9.8) was added, and after 30 min 30 μL of 5 N NaOH was added to stop the reaction.

(6) Absorbances were measured on a plate reader (410-nm filter), and concentrations of clomazone were calculated automatically by using Immunosoft software on the basis of the concentrations of a set of standards included on each plate. During the validation phase of this research, data was calculated manually and is reported as percent inhibition of color development. The percent inhibition was calculated as

$$\% \text{ inhibition} = (1 - B/B_0) \times 100$$

where B is absorbance in the presence of and B_0 is absorbance in the absence of clomazone.

Cross-Reactivity Measurements. For cross-reactivity study, 2 mg/mL stock solutions of clomazone analogues or herbicides were made in methanol and diluted 1:1000 (2 μg/mL) with distilled water. These compounds were initially tested at 2 μg/mL (2 ppm). Those compounds showing good cross-reactivities were further appropriately diluted in distilled water and run at four serial (1:3) dilutions. The cross-reactivity was calculated by using the formula CR = $(A/C) \times 100$, where CR is percent cross-reactivity, C is the concentration of clomazone that causes 50% inhibition, and A is the concentration of clomazone analogue that causes 50% inhibition. The cross-reactivities were compared with clomazone (100%) standard.

Soil Extraction. Approximately 1 g of soil plus 4 mL of distilled water (total volume 5.0 mL) were thoroughly mixed in polypropylene tubes (17 × 100 mm) by vortex suspension, allowed to stand overnight, mixed again, and centrifuged at 12350g for 15 min. The soil supernatants without any further treatments were analyzed by ELISA. For comparison with GLC analysis, 1.0-mL aliquots of the aqueous extracts of soils spiked with technical grade clomazone were extracted with four 1.0-mL portions of hexane which were combined, concentrated under nitrogen, and adjusted to a final volume of 1.0 mL with hexane.

Table I. Intraassay and Interassay Variation of Clomazone Standards[a]

clomazone, ppb	intraassay variation (N = 6)			interassay variation (N = 3)		
	% inhibition (mean)	SD	% cv	% inhibition	SD	% cv
1.37	25.9	5.6	21.7	24.1	5.4	22.3
4.1	34.2	6.3	18.5	33.5	2.0	6.0
12.3	54.0	5.3	9.9	52.1	2.2	4.2
37.0	66.1	4.0	6.1	64.8	2.1	3.2
111.0	81.9	1.3	1.6	80.1	2.3	2.9
250	88.4	1.3	1.4	86.1	2.4	2.7

[a] Intraassay variation was estimated by using two plates that contained six sets of triplicate wells of each indicated concentration of clomazone. The percent inhibition of the standards compared to control (in the absence of clomazone) is reported. A set of standards (triplicate) run on three days was used to estimate interassay variation.

Bioassay. The greenhouse study was conducted by using bioassay and ELISA methods to determine the degradation rates of clomazone formulations in soil and compare the results of these two methods. Greenhouse soil into which 4EC and MEC formulations were incorporated at rates equivalent to 0.031–1.0 kg/ha was placed in pots. Soil subsamples were taken for ELISA analysis. Wheat and velvetleaf seeds were then planted in these pots. The pots were placed in a greenhouse and watered from the top initially and then subirrigated twice daily to avoid leaching of the herbicide from the soil. Two weeks after planting, discoloration (pigment bleaching) of the seedlings was visually evaluated by using a scale of 0–100%. This procedure was repeated on these same soils at 4-week intervals for 16 weeks. Between plantings the soil was allowed to dry out, the vegetation removed, and the soil broken up. The same species were replanted at 4, 8, 12, and 16 weeks after initial treatment.

A similar single-time planting bioassay was carried out with 108 soil samples collected from soybean fields treated with commercial clomazone formulations (4EC and 6EC) at rates of 0.84–1.4 kg/ha.

RESULTS

The reproducibility of the method based on a set of clomazone standards run on different plates on the same day (intraassay) and on different days (interassay) is summarized in Table I. The intraassay coefficient of variation (cv) of the standards used for the calibration of the assay ranged from 1.5 to 10% for standards between 12.3 and 250 ppb. At the lowest standard concentration of 1.37 ppb, the cv increased to 22%. Standards equivalent to 6.85–1250 ppb in soil after the extraction volume was adjusted for (actual concentrations from 1.37 to 250 ppb) were run on each plate. Over this range of clomazone concentrations the coefficient of correlation between the log concentration and percent inhibition was 0.97 or greater by using linear regression. Due to high variability with the low (1.37 ppb) standard, the sensitivity of the assay was set at 2 ppb (10 ppb in soil). The detection limit was at least 1 ppb under these assay conditions.

The precision of the assay was estimated by using the known clomazone controls of 10 and 100 ppb which were routinely run on each plate. The intraassay coefficient of variation was 16.7% at 10 ppb and 19.4% at 100 ppb; between assay variations were 12.8% and 8.5%, respectively. This variability is composed of the variability in the set of standards as given in Table I plus the variability inherent in measuring the concentrations of clomazone in the control solutions.

The commercial 4EC formulation of clomazone was dissolved in water to give concentrations of active ingredient similar to the set of calibration standards routinely used in ELISA assay. Both sets of solutions were run on
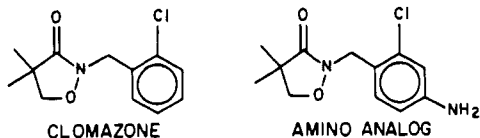
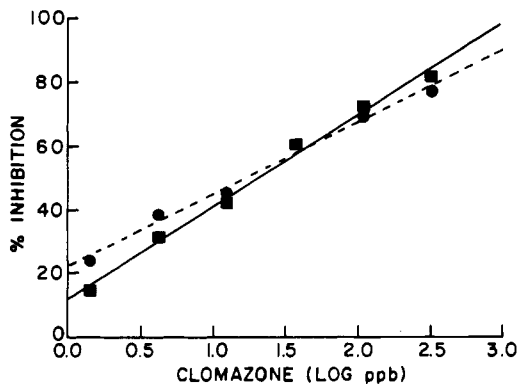**Figure 1.** Structure of clomazone and amino analogue.

Figure 2. Comparison of technical and formulated clomazone measured by ELISA. Technical clomazone (●) and Command 4EC herbicide (■) were diluted as described under Materials and Methods to give the indicated concentrations. Both sets of diluted compounds were run on the same plate. The regression equations are (●) $y = 22.1X + 22.8$ and (□) $y = 28.4X + 12.6$.
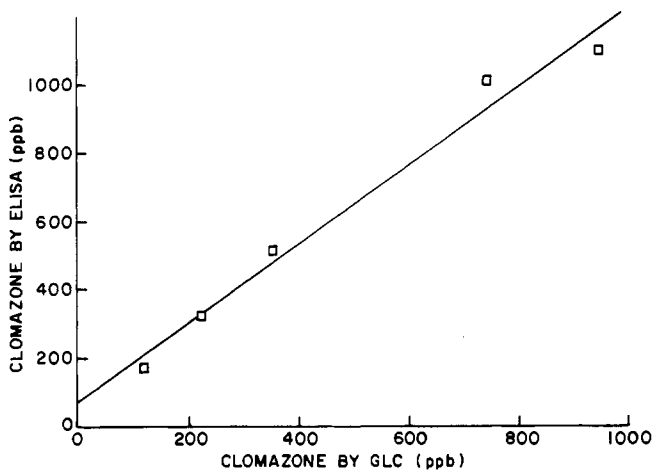

Figure 3. Correlation of ELISA and GLC analyses measurements of clomazone. Five replicate extracts of soil spiked with clomazone at concentrations of 0.1, 0.2, 0.4, 0.8, and 1.0 ppm were analyzed by ELISA and by GLC as described under Materials and Methods. The means of each set of five replicates are compared by linear regression with $y = 1.29X + 76.12$ and an $r^2 = 0.98$.

the same plate in the ELISA procedure, percent inhibition of color development was calculated, and the results were compared by linear regression (Figure 2). Except for the small bias in the slopes of the curves, there was no significant difference between the two curves, indicating the inert ingredients in the commercial formulation did not interfere in the assay.

Correlation with GLC Analysis. Soil samples spiked with clomazone at rates of 0.5–5.0 μg/g of soil were extracted with water. These soil extracts were either analyzed directly by ELISA or extracted with hexane which was then analyzed by GLC. The linear regression analysis of these results ($Y = 1.29X + 76.1$, $R^2 = 0.98$) indicates that the ELISA results are comparable with results obtained by GLC analysis (Figure 3). Each data point in Figure 3 represents an average value of five spiked samples. The standard deviation (SD) of ELISA was between 0.01 and 0.1, whereas for GLC analysis the SD was between
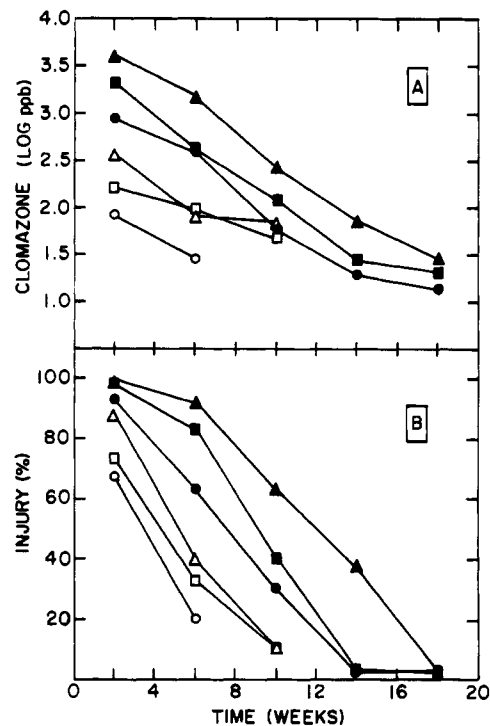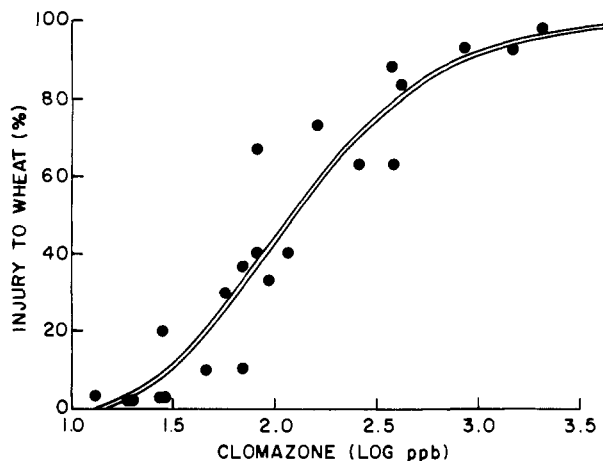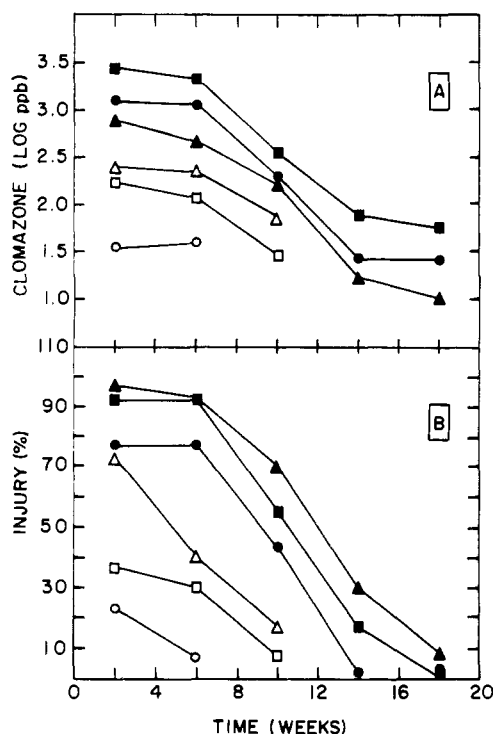

Figure 4. Change of soil concentration of clomazone and injury to wheat with time following treatment with an emulsifiable concentrate formulation. As described under Materials and Methods, soil treated with 0.031 (○), 0.0625 (□), 0.125 (△), 0.25 (●), 0.50 (■), or 1.0 (▲) kg/ha Command herbicide 4EC was analyzed for clomazone by ELISA (A) and bioassayed with wheat (B) at the indicated intervals.

0.02 and 0.18. Somewhat higher values and lower SD obtained with the ELISA compared to the GLC method can partly be accounted for by some loss of clomazone residues in the extra extraction step (with hexane) involved in the sample preparation for GLC analysis.

Comparison of Clomazone Content in Soil with Plant Injury. The results of a carryover experiment in which ELISA analysis and bioassay were performed at 4-week intervals on spiked soil samples are shown in Figure 4. The loss of immunoreactive clomazone followed first-order kinetics at all concentrations tested and was consistent with previous studies of the degradation of herbicides of short persistence in soil (Hurle and Walker, 1980); within experimental error the rates of degradation were the same at all concentrations. The decrease in immunoreactive clomazone (Figure 4A) was accompanied by a progressive decline in injury (Figure 4B). Except during the first 6 weeks at the two highest concentrations when the residual concentration of clomazone was near the threshold to cause complete bleaching, the decline in injury was linear with time. The data in Figure 4 were combined in Figure 5 to construct a dose–response curve. Thus, the expected sigmoid relationship was obtained (Figure 5); 50% injury (ED_{50}) occurred at a clomazone concentration of about 100 ppb. By use of a similar protocol, the correlation of ELISA analysis to bioassay injury was also demonstrable with microencapsulated (MEC) clomazone formulation (Figure 6). The slow release from the microcapsules delayed the onset of the first-order degradation by about 4 weeks (Figure 6A) compared with the 4EC formulation (Figure 4A). Associated with the slow release of clomazone there was also greater injury to the wheat seedlings (Figure 6B) compared to that observed with the 4EC formulation (Figure 4B). This time lag in concentration decline with MEC formulation can be explained by the slow release of active ingredients from

Figure 5. Injury to wheat caused by clomazone residues in soil. The curve was constructed from the data contained in Figure 4. The function $Y = A\{\log (X + 1)/B\}^C/1 + \{\log (X + 1)/B\}^C$ was used to fit dose–response data. X is dose, Y is percent injury to wheat, and A, B, and C are parameters.
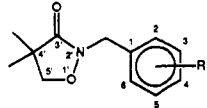

Figure 6. Change of soil concentration of clomazone and injury to wheat with time following treatment with a microencapsulated formulation. The procedure is as described in Figure 4 except a microencapsulated formulation was used.

the MEC capsules. The ED_{50} for the MEC formulation estimated from a dose–response curve constructed with the data in Figure 6 was about 100 ppb and was similar to the ED_{50} for the 4EC formulation extrapolated from Figure 5. Similar results were also obtained with velvetleaf (data not shown).
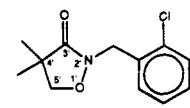
In a separate experiment, clomazone residues were measured by ELISA in 108 soil samples taken from 20 fields treated with the EC formulations of clomazone. These soil samples were planted with wheat, and injury was estimated 2 weeks after planting. The data were combined, and an attempt was made to construct a dose–response curve, similar to the procedure used with the data in Figures 4 and 5. Compared to the greenhouse study there was more scatter in this set of data, which prevented our obtaining a dose–response curve using standard higher order fit function (data not shown). Some

Table II. Cross-Reactivities of Compounds with Variation in Substitution in the Phenyl Ring

| compd | \multicolumn{5}{c}{phenyl ring R-substitution} | % cross-reactivity |
	2	3	4	5	6	
1	Cl	H	H	H	H	100
2	F	H	H	H	H	19
3	OCH$_3$	H	H	H	H	6
4	H	H	H	H	H	1.2
5	Cl	H	H	H	Cl	2.6
6	Cl	H	H	H	OH	0.4
7	Cl	H	H	OH	H	21.0
8	Cl	H	NH$_2$	H	H	96
9	Cl	H	COOH	H	H	214
10	Cl	H	OCH$_2$COOH	H	H	469
11	Cl	H	NHNH$_2$	H	H	128
12	Cl	H	OH	H	H	210
13	Cl	H	NHCOtBu	H	H	13
14	H	H	Cl	H	H	3
15	H	OCH$_3$	OCH$_3$	OCH$_3$	H	0.3

Table III. Cross-Reactivities of Compounds with Variation in 4,4-Dimethyl-3-isoxazolidinone Ring

| compd | \multicolumn{4}{c}{isoxalidinone ring substitution} | % cross-reactivity |
	2	3	4	5	
1	CH$_2$	O	(CH$_3$)$_2$	H$_2$	100
16	CH$_2$	S	(CH$_3$)$_2$	H$_2$	6
17	CH$_2$	O	CH$_3$,H	H$_2$	3
18	CH$_2$	O	(CH$_3$)$_2$	OH	0.9
19	CH$_2$	OH, H	(CH$_3$)$_2$	H$_2$	0.6
20	CH$_2$	O	(CH$_3$)$_2$	OCH(CH$_3$)$_2$	0.4
21	CH$_2$	O	(CH$_3$)$_2$	C$_6$H$_5$	ND^a
22	CH$_2$	H$_2$	(CH$_3$)$_2$	H$_2$	ND^a
23	CH$_2$	H$_2$	(CH$_3$)$_2$	O	4
24	CH$_2$	S	(CH$_3$)$_2$	O	ND^a
25	CHOH	O	(CH$_3$)$_2$	H$_2$	0.2

^{a} Nondetectable at 2 ppm.

of the scatter in these data can be attributed to differences in soil sampling and soil type and the presence of other chemicals in the fields. There was no attempt to identify and eliminate from the analysis soil samples that might have been treated with chemicals in addition to clomazone, although several symptoms such as poor seed germination and stunting suggested the presence of such chemicals in some soils.

Cross-Reactivity. Several compounds structurally related to clomazone were tested for cross-reactivity to the anti-clomazone antibody. The cross-reactivity results are summarized in Tables II–IV. The cross-reactivities of several analogues variously substituted on the phenyl ring are compared in Table II. Substitution of the 2-Cl by OCH$_3$ (3) or H (4) resulted in loss of nearly all binding, and 80% of the binding was lost by replacement with F (2). Analogues with 6-Cl (5) or 6-OH (6) lost, respectively, 97% and 99% binding. The 5-OH analogue (7) bound to the antibody about 20% as tightly as clomazone. The binding to the antibody of several analogues substituted at C-4 was equal to or greater than that of clomazone. Thus, except for 4-butylamine (13, 13% binding), the relative binding of the 4-amino (8) and 4-hydrazino (11) compounds was, respectively, 96% and 128% and of the 4-carboxy (9), 4-acetoxy (10), and 4-hydroxy (12) compounds was, respectively, 214%, 469%, and 210% with

Table IV. Cross-Reactivities of Analogues with Replacement of Isoxazolidinone Ring

Compound	Structure	% Cross-reactivity
26		ND*
27		57
28		25
29		102
30		0.5
31		1.3
32		ND*

*Nondetectable at 2 ppm. A =

Table V. Structures of Herbicides without Measurable Cross-Reactivities to Clomazone Antibodies

Metribuzin   Alachlor   Trifluralin   Norflurazon

Acifluorfen-sodium   Cyanazine   Butylate   Paraquat

Chlorsulfuron   Imazaquin   Atrazine   Metalachlor

Chlorimuron   Triallate   EPTC

Glyphosate   Bromoxynil   Linuron

respect to the binding of clomazone (1) to the antibody. The 4-amino analogue was used to prepare immunogen, and this may be the reason why a number of compounds substituted at this same position exhibit high cross-reactivity to the antibody.

The retention of significant binding with several different substitutions on the phenyl ring contrasts with what is observed when the isoxazolidinone ring is substituted. Generally any modification to the isoxazolidinone ring caused dramatic decreases in binding (Table III). The 6% binding was retained when the keto oxygen at 3' was replaced with sulfur (16), but when it was replaced with hydroxyl (19) or hydrogens (22), very little or no binding was detected. Replacing one of the two methyl groups at 4' with hydrogen (17) resulted in 3% retention of binding. It is interesting that an analogue (23) in which the oxygen at 3' and the pair of hydrogens at 5' are interchanged had 4% binding. An analogue with a hydroxyl on the bridging methylene moiety (25) did not bind at all to the antibody. Bulky substitutions like the isopropoxy and phenyl ring at 5' (20 and 21, respectively) also caused a complete loss of binding.

As shown in Table IV, expanding the isoxazolidinone ring to six members (30) or decreasing it to four members (31) caused nearly complete loss of binding. In contrast, three (27–29) of four compounds containing open-chain analogues of the isoxazolidinone ring showed significant affinity for the anti-clomazone antibody. A methylcarbamyl derivative (29) exhibited 100% binding. Interestingly, the only known major metabolite (26), a product resulting from reductive N–O bond cleavage of the parent compound in soil (Froelich et al., 1984) showed no detectable binding to the antibodies. Individual clomazone fragments, 2-chlorobenzyl alcohol and 4',4'-dimethyl-

3-isoxazolidinone, showed no detectable binding to anti-clomazone antibodies.

Eighteen commonly used herbicides representing different classes of chemistries which may be present in fields with clomazone were also tested for cross-reactivity with clomazone antiserum; none showed detectable binding at 2 ppm (Table V).

DISCUSSION

The ELISA method described here was developed to take advantage of the characteristics of quantitative assays based on immunological reagents. In particular, the present method can accommodate the routine analysis of a large number of soil samples for clomazone residues. Thus, using the ELISA procedure above with an automated plate reader, one person can process several hundred soil samples per week compared to less than 100 with a GLC method. The accuracy and the precision of the assay were adequate to study the dependence of biological activity on the concentration of clomazone residues in soil. The data presented above show that the within-run cv of the standard curve is between 1.5% at high concentrations of clomazone and 18% at the low concentrations, while the between-run cv is between 2.8% and 6% over the same concentrations. An estimate of the overall variability was obtained by routinely including clomazone controls (10 and 100 ppb) on the microtiter plates. At 10 and 100 ppb, the overall cv was between 8% and 19%.

The single aqueous extraction provides a basis for the efficient processing of a large number of samples with this procedure. It is likely that the fraction of clomazone in the soil which is biologically active is in the water phase and thus may be approximated by the fraction extracted into water. Clomazone that is tightly bound in soil is not likely to be biologically active. Parallel GLC and ELISA analysis of the aqueous extracts of spiked soil samples

gave similar results, indicating that clomazone extracted with water can be reliably measured by ELISA. The concentration of clomazone extracted from soil into water and measured by ELISA is an accurate indicator of biological effects, i.e., pigment bleaching. The utility of the ELISA for the detection and quantification of clomazone in soil is illustrated by those experiments in which biological activity was also measured. In greenhouse experiments, the rate of degradation of clomazone in soil can be conveniently measured by using ELISA. The ELISA analysis differentiated the availability of a active ingredient between two types of formulations (the 4EC and the MEC). The decline in injury with time at various application rates of clomazone formulations can be explained by the progressive disappearance of extractable clomazone. Degradation and tight binding to soil are probable explanations for this disappearance of clomazone with time.

An attempt was made to extend the utility of the ELISA to field soils. Although there was a good correlation between ELISA analysis and bioassay results under controlled greenhouse conditions, the correlation of ELISA to bioassay results in the soil samples taken from actual field applications was not clear-cut. This poor correlation between observed clomazone residues and bioassay injury in field samples can be explained by other factors such as soil sampling, soil type, lag time between application and sampling, and presence of other herbicides in the field. The need to understand these various factors illustrates the difficulty in translating an ELISA assay from controlled laboratory conditions to less controlled field conditions.

In the study to determine the cross-reactivities of compounds with structures related to clomazone, several were as reactive as, or more reactive than, clomazone. Animals were immunized with a conjugate prepared from an analogue containing an amino group on the phenyl ring at position 4, which is para to the isoxazolidinone ring. Several analogues substituted at position 4 on the phenyl ring bind equally well or better than clomazone (100%). These include the analogues with an amino group (96%), two with free carboxyls (214% and 469%), a hydroxyl (128%), and a hydrazine (128%). The tight binding of these analogues is consistent with the concept of an antibody binding pocket [e.g., Marquart and Deisenhofer (1982); Amit et al. (1986)] which is relatively more specific for the isoxazolidinone moiety of clomazone compared to the substituted phenyl moiety. If there are polar sites fortuitously located on the surface of the antibody protein to which the polar substituent on a hapten can bind, then additional binding energy may be obtained which can result in an affinity for the antibody which is greater than that of clomazone. This can be explained as a result of the immune response in which the immunogen containing attached haptens is presented to the lymphocyte receptors with the only possible binding being to the exposed isoxazolidinone end because the phenyl end is covalently attached to the BSA and thus partially shielded. This would result in the formation of antibodies which are specific for the isoxazolidinone substructure. The higher specificity of antibodies toward the hapten substructure further away from the conjugated functional group has been previously reported (Weiler, 1983; Schwalbe et al., 1984; Barthe and Stewart, 1985). The data presented here indicate that this substructure includes the isoxazolidinone ring, the methylene bridge, and the 2- and 6-positions on the phenyl ring. If the higher binding of analogues with polar groups at position 4 on the phenyl ring is due to ionic interactions, then binding should be highly de-

pendent on degree of ionization of the charged group. Binding should, therefore, be highly dependent on pH.

CONCLUSION

We have developed a sensitive, specific, and convenient one-step aqueous extraction ELISA procedure for analyzing the clomazone levels in soil. The ELISA analysis of spiked soil samples showed good correlation not only with GLC analysis but also with plant injury in a greenhouse bioassay. Our studies demonstrate that ELISA analysis can be used to study the persistence of herbicides in the soil under controlled greenhouse conditions and possibly in the field, if consideration is given to other potentially interfering factors relevant to field trials such as soil sampling, soil type, irrigation, method of herbicide application, time of injury assessment, and presence of other herbicides in the field.

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