Chlorsulfuron Determination in Soil Extracts by Enzyme Immunoassay

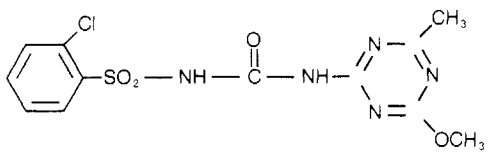
Marian M. Kelley, Edward W. Zahnow,* W. Christian Petersen, and Stephen T. Toy

Chlorsulfuron is the active ingredient in Glean Herbicide, a herbicide used to control weeds common to cereal crops. The harmful effects of very low concentrations in soil on broad-leafed crops make it important to be able to determine the chlorsulfuron concentration in soil. An enzyme-linked immunosorbent assay (ELISA) has been developed which provides a rapid and efficient method of quantitating nanogram levels of chlorsulfuron in crude soil extracts. Compounds related to chlorsulfuron, other sulfonylurea cereal herbicides, and soil extracts of chlorsulfuron were examined for their ability to inhibit the binding of rabbit antibody to the chlorsulfuron antigen. The assay detects some sulfonylurea herbicides which are chemically similar to chlorsulfuron, but it does not detect certain other sulfonylurea herbicides or the primary degradation products of chlorsulfuron. The ELISA combines the advantages of chemical and physical procedures in identifying herbicidal substances with the advantages of biological assays in identifying biologically active materials.

The application of immunoassay methods to the analysis of pesticides has been treated in great detail by Hammock and Mumma (1980). Immunochemical methods have also been applied to the analysis of benomyl by Newsome and Shields (1981), diflubenzuron and BAY SIR 8514 by Wie et al. (1982) and Wie and Hammock (1982), diclofop-methyl by Schwalbe et al. (1984), paraquat by Levitt (1977, 1979) and Fatori and Hunter (1980), parathion by Ercegovich et al. (1981) and Vallejo et al. (1982), paraoxon by Hunter and Lenz (1982), and 2,4-D and 2,4,5-T by Rinder and Fleeker (1981).

Sulfonylureas, as described by Levitt (1978), represent a new class of herbicides, and although the paper of Kajinuma et al. (1982) reports a radioimmunoassay for the analysis of a sulfonylurea in serum, the work reported below extends the application of immunoassay techniques to the residue analysis of the sulfonylurea herbicide, chlorsulfuron, in soil.

Chlorsulfuron, the active ingredient in Glean Herbicide is used to control weeds common to wheat and other cereal crops (Levitt et al., 1981). Very small residual amounts



Chlorsulfuron

of chlorsulfuron in soil may be injurious to broad-leafed rotational crops such as sugar beets and sunflowers (Palm, 1980). Therefore, it is necessary to have an efficient and rapid assay system to determine chlorsulfuron concentrations in soil samples. At present, the usual method for analysis of chlorsulfuron in soil is HPLC with photoconductivity detection, but these determinations require extensive sample preparation and cleanup steps (Zahnow, 1982). Current bioassays, using root (Hsiao and Smith, 1983) or lettuce shoot growth (Bond and Roberts, 1976) are time consuming and cumbersome. This report describes the enzyme-linked immunoassay (ELISA), a sensitive immunological technique that can monitor nanogram quantities of chlorsulfuron in crude soil extracts. The ELISA technique is based on the ability of animals to produce highly specific antibodies to foreign materials. Antibody is collected from rabbit serum and becomes one reagent in a rapid, solid-phase assay that is both specific and sensitive (Engvall and Perlmann, 1971; Van Weeman and Schurs, 1971).

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EXPERIMENTAL SECTION

Conjugate Preparation. Chlorsulfuron conjugated to either keyhole limpet hemocyanin (KLH) or to bovine serum albumin (BSA) was prepared. Briefly, *N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-4-amino-2-chlorobenzenesulfonamide (0.5 g, 1.34 mmol) was slurried in a mixture of acetic acid (10 mL) and propionic acid (5 mL) and cooled to 0 °C. To this solution was added concentrated hydrochloric acid (0.42 mL) and sodium nitrite (0.1 g, 1.5 mmol) and the temperature held at 0 °C for 0.5 h. The light yellow opalescent solution was then divided into two portions (7.5-mL each) and used immediately to prepare the conjugates.

One portion of the resulting chlorsulfuron diazonium solution (7.5 mL) was slowly added to keyhole limpet hemocyanin (1.0 g, Calbiochem, lot 130072) in 40 mL of a buffer solution, described by Clarke and Cassals (1958) of 0.05 M borate and 0.12 M sodium chloride. The slow addition of the diazonium solution was accompanied by addition of 1 N sodium hydroxide solution to maintain pH 9.0. The final yellow solution was stored at 4 °C.

The other portion of the chlorsulfuron diazonium solution (7.5 mL) was slowly added to bovine serum albumin (1.0 g, Calibiochem, lot 203576) in a borate buffer solution (40 mL of 0.05 M borate and 0.12 M sodium chloride). The slow addition of the diazonium solution was accompanied by addition of the 1 N sodium hydroxide solution to maintain pH 9.0. The final yellow orange solution was stored at 4 °C.

Antibody Preparation. Two 3-lb female New Zealand rabbits (Hazleton) were each injected with a mixture of 1 mg of chlorsulfuron/KLH and 1 mL of complete Freund's adjuvant. Similarly, two other rabbits were each injected with a mixture of 1 mg of chlorsulfuron/BSA and 1 mL of complete Freund's adjuvant. Intravenous (I.V.) boosts were done 2 months later with 0.1 mg of the chlorsulfuron/KLH or chlorsulfuron/BSA conjugate. I.V. boosts were performed every 3 months thereafter, as titers decreased. Whole blood was taken weekly and immune sera collected and stored at −20 °C until needed. The

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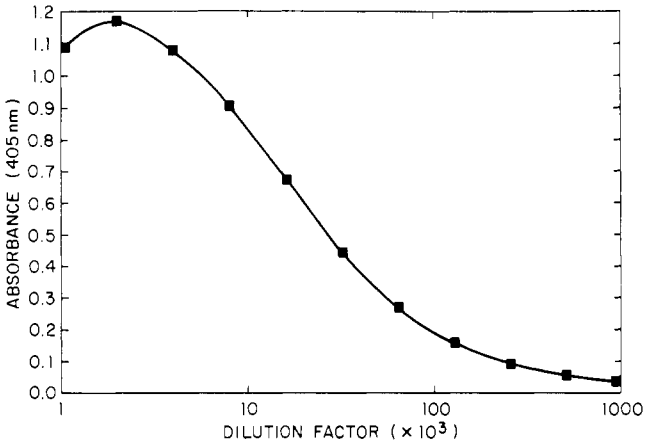


Figure 1. Dose response curve of immune rabbit serum.

conjugate not used for the immunization was used as the immobilized antigen to prevent interfering cross-reactivities between carrier proteins.

The antibodies from all four rabbits demonstrated comparable activity in the ELISA screen, as did pools of the antibodies produced by the pair of rabbits injected with either the KLH or BSA conjugates of chlorsulfuron. To standardize the assay, one bleed of serum from a rabbit which had been injected with the chlorsulfuron/KLH conjugate was arbitrarily selected.

Immunoassay. Chlorsulfuron/BSA (300 ng) was passively adsorbed to a 96-well polystyrene microtiter plate (Costar EIA no. 3590) in phosphate buffered saline (Gibco) at 4 °C overnight. Unbound antigen was aspirated, and the plate washed with a Dynatech Miniwash. Unoccupied sites were blocked with 0.05% Tween-20 (Sigma) in PBS (PBS/Tw). Rabbit antibody titers were established. Serial dilutions of immune sera in PBS/Tw were prepared, and 200 μL was incubated with the bound antigen for 1 h at ambient temperature. Unbound rabbit serum was washed away, and goat anti-rabbit IgG, conjugated to alkaline phosphatase (Tago Co.), was incubated with the antigen/antibody conjugate for 1 h at ambient temperature. All wells were again washed with PBS/Tw, and p-nitrophenylphosphate substrate (Sigma) was added at 1 mg/mL in diethanolamine buffer (Sigma) pH 9.8. Color development was determined quantitatively at 405 nm by the Dynatech MR580 Microelisa Auto Reader.

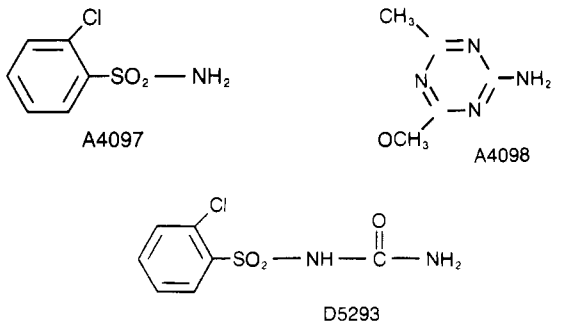
Inhibition Studies. Equal volumes of a known concentration of free chlorsulfuron and a dilution of immune rabbit antisera were incubated for 1 h at ambient temperature. The mixture was then allowed to incubate on the immobilized antigen plates for 1 h at ambient temperature. Only unbound antisera can bind to antigen. Once incubated with enzyme-labeled second antibody and substrate, color development occurs in direct proportion to the amount of unbound antibody available and in indirect proportion to the original concentration of free chlorsulfuron.

In a series of experiments, chlorsulfuron was replaced with known concentrations of related compounds, various Du Pont-produced sulfonylurea herbicides, and finally with known concentrations of chlorsulfuron in soil extracts.

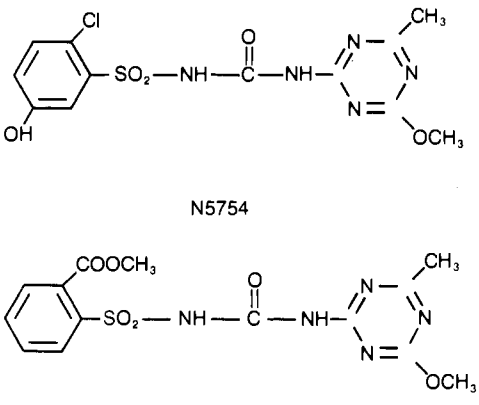
RESULTS AND DISCUSSION

In a standard solid-phase ELISA assay, a dose response curve was demonstrated (Figure 1) when a fixed concentration (300 ng) of chlorsulfuron was adhered to polystyrene wells and serial dilutions of rabbit immune sera were added. The rabbit sera exhibited reactivity when they were diluted as much as one to one million. Therefore, the antibody binding capacity of chlorsulfuron has been demonstrated. To make comparisons in future experiments, a single bleed (10/25/83) of the rabbit was aliquoted in 100-μL quantities, frozen, and used in the remaining experiments.

A series of experiments was performed to determine if competitive antigen would bind to the antibody in the antibody/antigen reaction described and inhibit further binding. Immune serum was incubated with free chlorsulfuron. Free chlorsulfuron should possess the antigenic determinants (or epitopes) which are recognized by the rabbit antibodies and which confer its specificity. In the appropriate antibody/antigen concentration range, binding occurs in increasing percentage as the free antigen increases or as the specific antisera decrease. This can be demonstrated in an indirect manner by exposing the bound rabbit antisera to a fixed amount of adhered chlorsulfuron. Control immune antisera, not exposed to free chlorsulfuron, bind in the usual dose-dependent manner. However, antisera incubated with a known amount of chlorsulfuron have fewer sites available to bind to immobilized chlorsulfuron. Enzyme-linked second antibody binds to the available rabbit antibody, and proportional color development occurs. Therefore, chlorsulfuron can be used as an inhibitory antigen and can be identified by ELISA.



Cross-reaction competitive assays were performed to identify which chlorsulfuron degradation products and which related compounds would be recognized by rabbit anti-chlorsulfuron antibody. When chlorsulfuron is broken down into three fragments, the ability to inhibit rabbit binding is lost. Inhibition curves of chlorsulfuron (DPX-



W4189) and these three degradation products are shown in Figure 2. Two related compounds whose bridge and heterocycle structures are the same as that of chlorsulfuron retain antibody binding activity (Figure 3). However, herbicidally active compounds, DPX-T5648 and DPX-F5384, in which the heterocyle structure has been modified or where the bridge has been lengthened by a methylene group, exhibit much less cross-reactivity. It would appear

Table I. Soil Properties

sample	type	pH	sand USDA, %	silt USDA, %	clay USDA, %	organic content, %
1	silty clay loam (Jones, OK)	6.9	8	56	36	3.1
2	silt loam (Rogers, ND)	8.0	43	51	6	4.8
3	silt loam (St. John, WA)	7.1	13	69	18	3.5

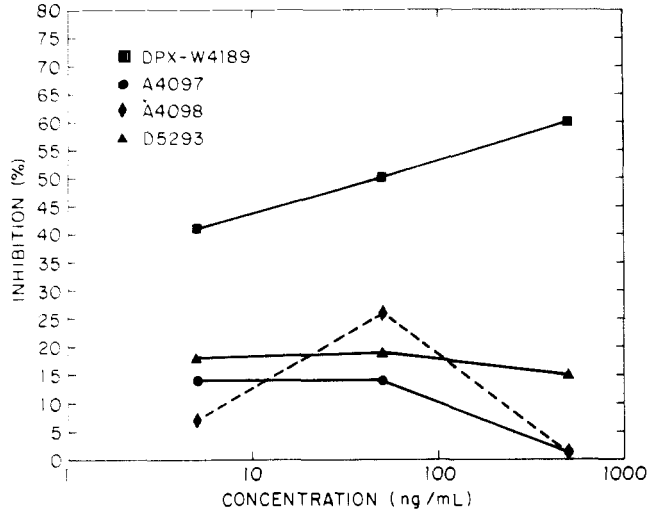


Figure 2. Competitive inhibition by free chlorsulfuron and degradation products of chlorsulfuron.

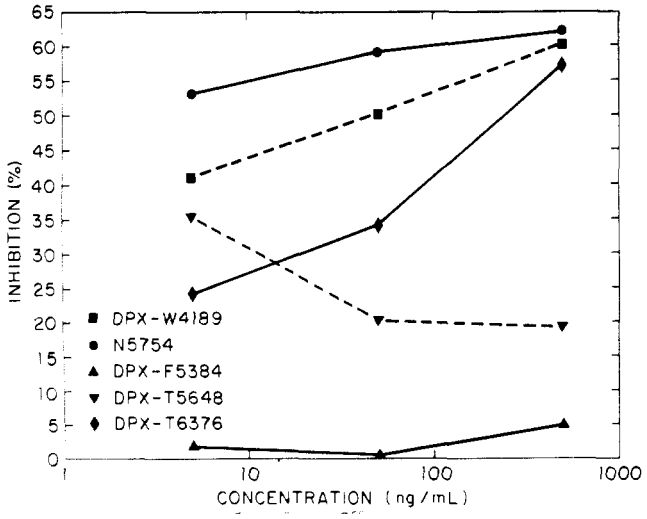
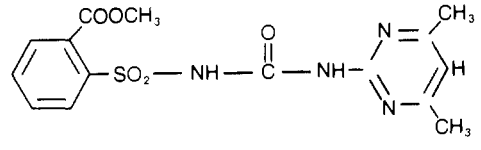
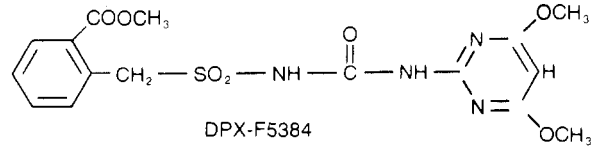


Figure 3. Competitive inhibition by sulfonylureas.

that these changes in structure cause alterations in spatial orientation which prevents ab/ag binding (Figure 3).



Sulfometuron Methyl



DPX-F5384

Finally, studies were done to determine if soil extracts obtained in the same manner as for HPLC analysis, would contain interferences which would make it impossible to identify chlorsulfuron in the assay. Aqueous alkaline extracts of three types of soil (Table I), with and without chlorsulfuron, were tested in the competitive inhibition assay. While soil extracts did contribute some inhibitory activity, soil with chlorsulfuron added demonstrated increased inhibition (Figure 4). In this figure all experimental data has been corrected for background inhibition. Extraction parameters, presently employed in HPLC analysis were examined. Adjusting the pH of the carbonate buffer (0.1 M sodium bicarbonate, 0.1 M sodium carbonate) from 10 to 7 did not affect the assay. Omitting a final filtration step also did not seem to interfere with the ELISA assay (data not shown).

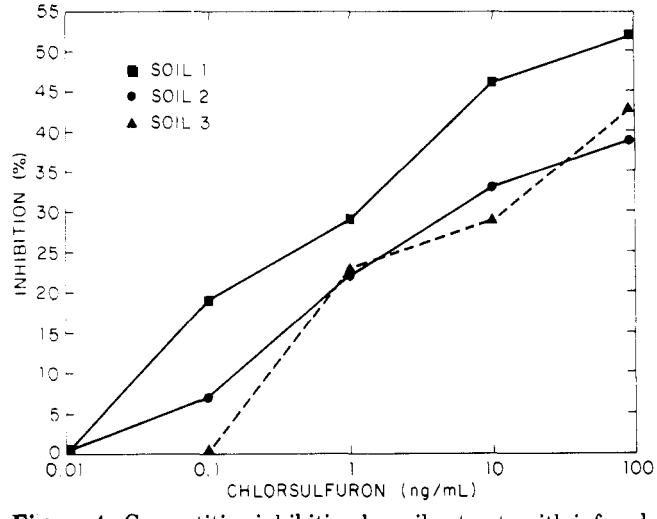


Figure 4. Competitive inhibition by soil extracts with infused chlorsulfuron.

These results show that chlorsulfuron can be measured at very low concentrations in extracts of soils which have widely diverse properties. With soils 1 and 2 the limit of detection for chlorsulfuron is 0.1 ng/mL (0.4 ppb in the soil) whereas with soil 3, the detection limit is about three times larger (1.2 ppb). These limits compare favorably with the corn root bioassay (0.1 ppb) and the HPLC method (0.2 ppb) for several reasons. First, the HPLC and bioassay approaches may encounter interferences which either raise the detection limits substantially or prevent any measurement at all. Second, the immunoassay is rapid and relatively inexpensive (50 samples/day) vs. the HPLC method (4 samples/day) or the bioassay which requires a week of plant growth. Third, the immunoassay is relatively specific in contrast to a bioassay which will respond to a wide variety of herbicidally active compounds. Positive identification of any compound is only possible through the use of complementary analytical methods (mass spectrometry, immunoassay, bioassay, HPLC) and a knowledge of the treatment history of the soil itself.

The fraction of biologically active chlorsulfuron in any specific sample of chemically extracted chlorsulfuron is a function of the soil type as well as the species of plant because of the complex effects of such variables as soil moisture and temperature. Other experiments have shown (Zahnow, 1982; Zahnow, 1985) that the HPLC and corn root bioassay measurements on field-treated, aged soils are very similar. Since the HPLC extraction procedure is used to obtain samples for the immunoassay, the correlation

with bioassay measurements is made indirectly.

CONCLUSION

Experiments cited in this paper indicate that chlorsulfuron and chemically similar compounds in unfiltered soil samples can be detected in nanogram concentrations by ELISA. Further work must be done to optimize the assay. To this time, crude immune serum from one rabbit has been used as the source of antibody. Pooling several animals' sera and even several bleeds from the same animal may enhance the assay's sensitivity. Furthermore, IgG fractions of the rabbit antibody can be isolated with a minimum effort by using commercially available immunoaffinity columns which may further increase sensitivity. The competitive binding assay described in this paper relied on an initial binding which approached saturation of the antibody molecule. Greater flexibility may be achieved if the competition and binding are permitted at the same time since it is more likely to result in a linear dose/response inhibition curve. Preliminary results indicate that chlorsulfuron in soil extracts can be calculated from a standard curve at concentrations as low as 0.1 ng/mL. This corresponds to a concentration of chlorsulfuron in soil of 0.4 ppb. Taken together, these results indicate that the ELISA is a promising immunological tool which may apply directly to herbicide analysis in soil.

Registry No. DPX-W4189, 64902-72-3; A4097, 6961-82-6; A4098, 1668-54-8; D5293, 5051-77-4; D5754, 81123-38-8; DPX-F5384, 96081-37-7; DPX-T5648, 74222-97-2; DPX-T6376, 74223-64-6.

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Gas Chromatographic Determination of 3,6-Dichloropicolinic Acid Residues in Soils and Its Application to the Residue Dissipation in a Soil

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Residues of 3,6-dichloropicolinic acid (3,6-DCP) were extracted from soils with sodium hydroxide, partitioned in ether, methylated by diazomethane, and determined by GLC-^{63}Ni ECD with a fused silica capillary column. Recoveries from four types of soil range from 84.5 to 94.3% for fortified samples at 0.05–2.5 mg/kg. The method was applied to the determination of residue dissipation in a soil. 28 days after the field treatment at a rate of 360 g of 3,6-DCP/ha the limit of detection (0.05 mg/kg) was achieved.

INTRODUCTION

Previous papers have described a method for the determination of 3,6-dichloropicolinic acid (3,6-DCP) in sugar beets by gas-liquid chromatography (Galoux et al., 1982) and the residue dissipation of this herbicide after a field treatment in 1981 on a sugar beets crop (Galoux et al., 1983). Results have showed a light persistence of the pesticide at a rate of 0.11, 0.13, 0.17, and 0.27 mg/kg in the beets when Cyronal emulsifiable concentrate was applied at rates of 120, 150, 180, and 360 g of 3,6-DCP/ha.

The present paper explains the modification to the analytical procedure to use it for the determination in soil with a sensitivity of 0.05 mg/kg. It was applied on four types of soil, in laboratory conditions; but also to determine the residue dissipation of 3,6-DCP in soil during the extended experimentation in 1981 and to finish off the previous work on sugar beet crop of 1981.

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EXPERIMENTAL SECTION

Reagents. They are very similar to those described in the previous papers of 1982 and 1983. (a) Diazomethane. Prepared by reaction of *N*-methyl-*N*-nitroso-*N'*-nitroguanidine with sodium hydroxide and absorption in cool ether. An efficient fume hood and appropriate safety precautions should always be used when handling diazomethane and ether. (b) Standard. 3,6-Dichloropicolinic acid (purity >99%) was supplied by Dow Chemical Co. (Midland, MI.). Reference standard solutions for GLC (0.1–10 mg/L) were prepared in ether from a stock solution of 0.1000 g of standard 3,6-DCP in 250 mL of ether. For

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