Development of an Enzyme-Linked Immunosorbent Assay for Imazaquin Herbicide

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A sensitive immunoassay system was developed for the quantitation of imazaquin, the active ingredient for SCEPTER herbicide. The assay required a polyclonal antibody generated by immunizing rabbits with a bovine serum albumin conjugate of 5-(2-formylvinyl)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)benzoic acid. A competitive ELISA system employing horseradish peroxidase conjugated 6-isothiocyanatoimazaquin as a competing molecule was shown to be best for the quantitation of imazaquin. Tetramethylbenzidine was the color-developing substrate. A linear dose–response between 0.4 and 25 ng/mL imazaquin was demonstrated using the above reagents. Linear dose–response was also demonstrated for imazapyr (active ingredient for ARSENAL), imazethapyr (active ingredient for PURSUIT), and imazamethabenz methyl (active ingredient for ASSERT). The antibody was shown to be specific for the imidazolinone class of compounds; no response was observed with non-imidazolinone agrochemicals as well as some metabolites of imazaquin.

INTRODUCTION

The imidazolinone class of herbicides, imazaquin (SCEPTER), imazethapyr (PURSUIT), imazapyr (ARSENAL), and imazamethabenz methyl (ASSERT), are inhibitors of the enzyme acetohydroxy acid synthetase (AHAS) (Shaner et al., 1984). The structures of the compounds are depicted in Figure 1. AHAS enzymes are involved in branched-chain amino acid synthesis in the plants. Since AHAS enzymes are not present in mammals, fowl, or fish, the application of imidazolinone herbicides has no harmful impact on humans and the environment (Gagne et al., 1991).

The imidazolinones are applied either preplant or pre- or postemergence over the soil for the control of broadleaved weeds. Although imidazolinones normally degrade in the environment (Mangels, 1991), under drought conditions residues remaining in the soil need to be monitored since even very low levels of imidazolinone herbicide can affect certain sensitive rotational crops. The current analytical techniques for quantifying imidazolinones in soil or crop plant specimen are gas chromatography and high-performance liquid chromatography. As imidazolinone herbicides are more widely used, the demand for imidazolinone residue analysis should also increase. It would, therefore, appear desirable to develop a simple and rapid analytical method for quantitating imidazolinone residues to increase analytical capacity and provide a rapid turnaround time for sample analysis.

Since the review of Hammock and Mumma (1980) on immunoassay technology for agrochemicals, the volume of publications relating to immunoassay methods for analyzing fungicides, herbicides, and insecticides has increased dramatically (Harrison et al., 1988; Van Emon et al., 1989; Jung et al., 1989). Immunoassay technology is being demonstrated as a viable alternative for and a complement to traditional analytical methods for monitoring agrochemicals.

We report the development of a direct competitive enzyme immunoassay for the analysis of imazaquin. We shall present the synthesis of the immunogens, the enzyme conjugates, and the assay system. Data on the specificity of the antibody will also be included.

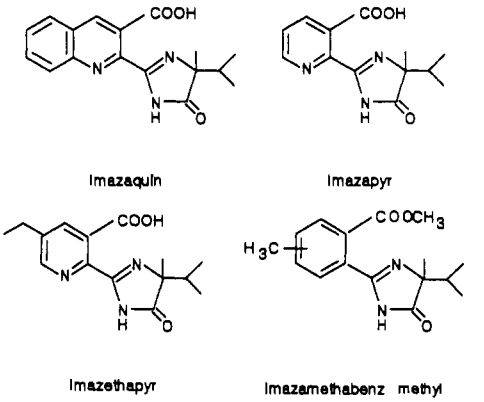

Figure 1. Structures of the imidazolinone herbicides.

MATERIALS AND METHODS

Materials. Analytical grade imazaquin, imazethapyr, imazamethabenz methyl, 6-nitroimazaquin, and metabolites of imazaquin were synthesized at the American Cyanamid Co. (Princeton, NJ). All other herbicides were of technical grade.

Bovine serum albumin and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase was from Boehringer Mannheim (Indianapolis, IN). Activated horseradish peroxidase hydrazide was from Molecular Probes, Inc. (Eugene, OR). Tetramethylbenzidine substrate was from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Non-fat dry milk was from Carnation Co. (Los Angeles, CA). HRP conjugated goat anti-rabbit IgG was from Organon Teknika Corp. (Durham, NC). Pyridinium chlorochromate and (triphenylphosphoranylidene)acetaldehyde were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). All other solvents and reagents were of the highest grade from J. T. Baker Co. (Phillipsburg, NJ). Polystyrene 96-microwell plates were from Costar (Cambridge, MA).

Instrumentation. A microwell reader (V_{max} reader) with SoftMax curve fitting software was from Molecular Devices Corp. (Palo Alto, CA). An Ultrawasher II microplate washer was purchased from Dynatech Laboratories, Inc. (Chantilly, VA). A ProPette automatic pipettor (Perkin-Elmer, Norwalk, CT) was used for dispensing liquid. The Waters 840 HPLC system (Waters Associates, Milford, MA), a TSQ 4600 Finnigan-MAT mass spectrometer (Finnigan, San Jose, CA), a Varian XL-300

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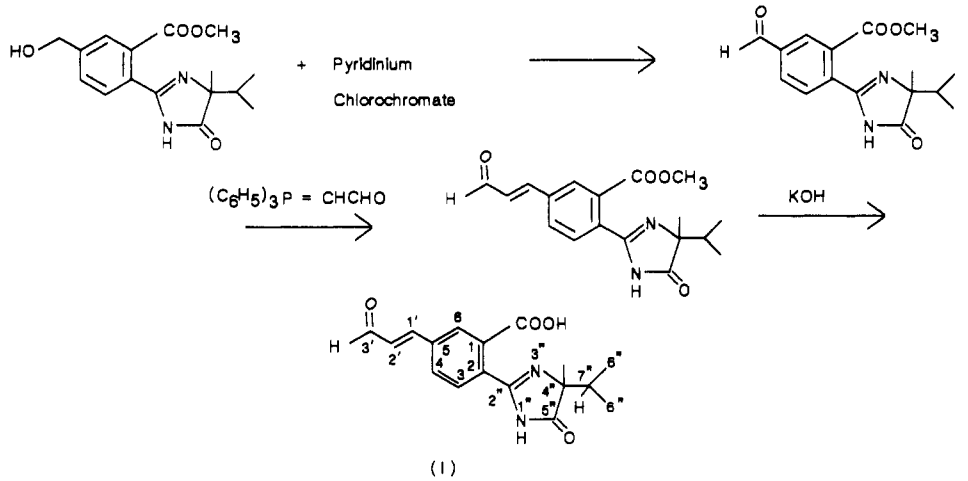


Figure 2. Synthetic route of compound I.

MHz NMR spectrometer (Varian, Sunnyvale, CA), and a Perkin-Elmer infrared spectrometer (Perkin-Elmer) were used for the purification and characterization of the haptens.

Hapten Synthesis and Verification. 5-(2-Formylvinyl)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)benzoic acid (I) was synthesized as outlined in Figure 2. Methyl α-hydroxy-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*m*-toluate was prepared at American Cyanamid from 4-carbomethoxybenzoyl chloride via a multistep sequence (T. Drabb, personal communication). A suspension of methyl α-hydroxy-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*m*-toluate (5.12 g, 16.86 mM) in methylene chloride (50 mL containing 4% pyridine) was stirred at room temperature under nitrogen with pyridinium chlorochromate (15.54 g, 72.08 mM) added in one portion. The reaction mixture was stirred for 2 h at room temperature and then quenched by adding ether. The mixture was filtered through a Florisil column and evaporated to a yellow oil. The oil was flash chromatographed on silica gel (200 mL) using ethyl acetate for elution. A colorless oil (1.88 g) was collected. The product was suspended in dry toluene with (triphenylphosphoranylidene)-acetaldehyde (2.05 g, 6.74 mM) and heated under reflux in an atmosphere of nitrogen for 4 h. The mixture was cooled to room temperature, and the solvent was evaporated. The resulting dark gum was flash chromatographed on silica gel (200 mL) and eluted with ether. The material was pooled and evaporated to yield a yellow foam. This material was characterized by ^{1}H NMR to be a mixture of olefinic products and triphenylphosphine oxide. The mixture was hydrolyzed without further purification by dissolving the product in a mixture of water (8 mL), 95% ethanol (2 mL), and 5 mL of 1 N KOH in methanol. The reaction mixture was heated under reflux in an atmosphere of nitrogen for 90 min. To the cooled mixture was added 10 mL of water, and the pH was adjusted to 2 with 1 N HCl. The solvents were evaporated to a dark brown residue. The material was dissolved in anhydrous ethanol (20 mL) and filtered to remove insoluble salts. The residue (1.1 g) obtained when the filtrate was evaporated was purified by C_{18} reversed-phase HPLC [semipreparative column (Dynamax column, 30 cm × 21.4 mm i.d., particle size 8 μm)]. The fractions were eluted with a methanol/water gradient from 25% to 100% methanol over 32 min at a flow rate of 13.2 mL/min. After evaporation of the solvents, the desired aldehyde was collected as a white powder (102.6 mg): mp 110–118 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.736 (d, 1 H, J = 7.2 Hz, C*H*O, H-3′), 8.336 (S, 1 H, Ar*H*, H-6), 8.013 and 7.679 (two d, 1 H each, J = 7.5 and 7.8 Hz, Ar*H*, H-3 and H-4), 7.782 (d, 1 H, J = 16.2 Hz, vinyl H, H-1′), 6.937 and 6.884 (d of d, 1 H, J = 7.2 and 15.9 Hz, vinyl H, H-2′), 2.07 (M, 1 H, H-7″), 1.412 (S, 3 H, C*H*$_3$-4″), 1.097 (d, 3 H, J = 6.3 Hz, 2 × C*H*$_3$-6″), 0.966 (d, 3 H, J = 6.3 Hz, 2 × C*H*$_3$-6″).

Preparation of Immunogen. Compound I was conjugated to a protein carrier bovine serum albumin (BSA) or ovalbumin (OA) through the hydrazide functions on the proteins. The protein hydrazides were prepared according to the method of O'Shannessy et al. (1987). Commercial preparations were used. The linkage between compound I and the BSA or OA hydrazide was effected by adding a 50–100-fold molar excess of aqueous solution of compound I in 0.1 M sodium phosphate buffer (pH 7.5) with 10% methanol to the protein in the same buffer solution at a final protein concentration of 10 mg/mL. The mixture was stirred at room temperature for 3 h followed by 18 h at 4 °C. The product was passed through a Sephadex G-25 column equilibrated with 0.05 M ammonium acetate buffer to remove excess salt and reagents. The protein fraction eluted at void volume was monitored by absorption at 280 nm. The pooled protein fractions were lyophilized and stored at −20 °C. The conjugation number was estimated by titration of the available amino groups with trinitrobenzenesulfonic acid in the presence of denaturing agent (Habeeb, 1966). Protein concentration was determined by dye bindings (Bio-Rad dye binding assay). Hapten numbers were found to be 35 for BSA and 12 for OA.

Antibody Production. New Zealand white rabbits were used. A solution of 0.5 mg of BSA conjugate in 1 mL of 0.1 M sodium phosphate buffer (pH 7.5) was emulsified with 1 mL of Freund's complete adjuvant. The emulsion was introduced perilymph nodally. At 3-week intervals, boosters containing 0.25 mg of protein conjugate in 0.5 mL of buffer emulsified with 0.5 mL of Freund's incomplete adjuvant were administered. Blood samples were collected via the ear vein 10 days after each boost. Sera from the clotted blood samples were monitored for the presence of antibody.

Horseradish Peroxidase Conjugate Preparation. An imazaquin analogue, 6-nitroimizaquin, was used for the conjugation to enzyme horseradish perxiodase (HRP). The reaction scheme is depicted in Figure 3. To a solution of 6-nitroimazaquin (212 mg, 0.594 mmol) in 100 mL of methanol was added a 50-mg quantity of 10% palladium on carbon under an argon atmosphere. The mixture was allowed to shake under a hydrogen atmosphere (4 psi) for 30 min at room temperature. The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. The concentrate was partitioned between 100-mL portions of chloroform and water, and the layers were separated. The organic layer was washed three times with water and dried over sodium sulfate, and the solvent was removed under reduced pressure to yield 170 mg of a yellow solid. The product was characterized by infrared spectrometry.

To a suspension of 170 mg of 6-aminoimazaquin in 25 mL of methanol was added 0.12 mL (1.56 mmol) of thiophosgene. The reaction was allowed to stir for 5 min at room temperature to yield a homogeneous solution. The solvent was removed under reduced pressure. The residue was redissolved in a small amount of methanol and filtered, and the filtrate was added to 25 mL of ether. The resulting precipitate was collected, washed with ether, and dried under reduced pressure to yield 190 mg of a brown powder. Infrared spectrometry was used for the identification of the isothiocyanatoimazaquin product.

Horseradish peroxidase conjugation to the isothiocyanato compound was effected by dissolving 20 mg of the enzyme and 3 mg of 6-isothiocyanatoimazaquin in 5 mL of 0.1 M sodium phosphate buffer (pH 7.5). The solution was stirred for 2 h at room temperature and then overnight at 4 °C. The conjugate

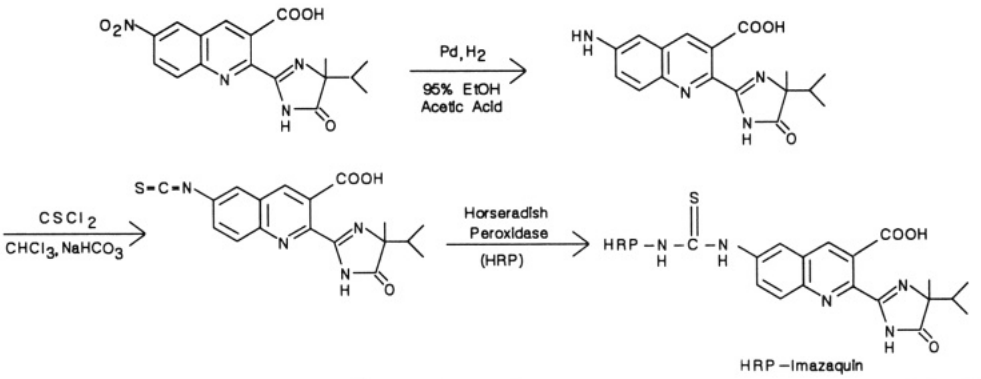


Figure 3. HRP conjugation to 6-nitroimazaquin. Conversion of the nitro function to amine was accomplished by reduction and treatment of the amino group with thiophosgene to form the isothiocyanato group. The isothiocyanato imazaquin was reacted with the amino groups of HRP.

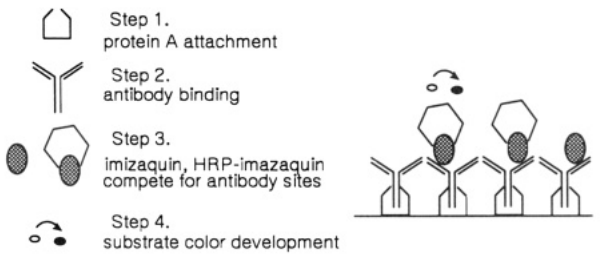


Figure 4. Competitive ELISA format.

was purified on a Sephadex G-25 column using 30 mM ammonium acetate buffer as eluant. The desalted enzyme fraction was pooled and lyophilized for storage at 4 °C.

Horseradish peroxidase conjugate to compound I was prepared according to the method reported by Murayama et al. (1978). Horseradish peroxidase at 1 mg/mL was prepared in 0.1 M sodium bicarbonate (pH 9.0). Compound I, also at 1 mg/mL, was dissolved in 10% aqueous methanol solution, pH adjusted to 9.0. A 40:1 molar ratio of compound I to HRP was mixed and stirred at room temperature for 2 h. To the mixture was added 5 mg of solid cyanoborohydride, and the resulting solution was allowed to mix at 4 °C for 4 h. The conjugation product was passed through a Biogel Econo Park 10DG column to remove excess reagent. The product was eluted in 0.1 M sodium phosphate buffer (pH 7.5) and filter sterilized through a 0.22-μm filter. It was stored at 4 °C.

Monitoring Serum Antibody. Serum antibody was detected by a double-antibody method using OA conjugate of compound I as the coating antigen. The antigen was coated on microwells at 4 °C overnight with 0.1 μg of protein per well in 0.1 M sodium carbonate buffer (pH 9.5). The wells were washed four times with 250 μL of 0.01 M sodium phosphate buffer (pH 7.6) containing 0.14% sodium chloride and 0.01% Tween 20 (PBS-T). The wells were blocked at room temperature with 5% milk solution prepared in 0.01 M sodium phosphate buffer (pH 7.6) with 0.14% sodium chloride (PBS) and washed as described above. Antibody was detected by incubating 100 μL of serum solution in the antigen-coated wells for 1 h. After four washes with PBS-T, 100 μL of HRP conjugate of goat anti-rabbit IgG was added followed by 30 min of incubation. After a wash to remove unbound HRP conjugate, 100 μL of freshly prepared substrate tetramethylbenzidine (TMB) was added. The amount of antibody present in the serum was directly proportional to the color intensity at 650 nm as obtained using the V_{max} reader. All assay incubations were carried out at room temperature.

Competitive Enzyme Immunoassay. For the purpose of generating a simple assay system amenable for adoption as an on-site assay, we developed a direct competition system using HRP-linked compounds as depicted in Figure 4. The assays were carried out by first coating the microwells with 100 μL of 0.1 μg of protein A in 0.1 M sodium carbonate buffer (pH 9.5) overnight at 4 °C. The contents were discarded and the wells washed four times with 250 μL of PBS-T. The wells were blocked with 5% non-fat milk solution in PBS for 30 min. After four washes with PBS-T as described before, they were stored sealed at 4 °C until use. One hundred microliters of antibody of appropriate dilution was allow to bind to the protein A coated microwells for 1 h at room temperature. The wells were again washed. The quantity of BSA precleared antibody as well as the HRP conjugate required for the assay was determined previously by a checkerboard test in which varying dilutions of antibody were reacted with varying dilutions of HRP conjugate on the protein A coated microwell. The dilutions which produced an optical density increase of 1.5 in 10 min were chosen for the assay.

To establish a dose–reponse curve for imizaquin, 50 μL of imazaquin solution of concentration between 50 and 0.8 ng/mL in PBS and 50 μL of the HRP conjugate in PBS with 1% BSA were added to the antibody coated wells. The solution was incubated for 30 min at room temperature. After washing, 100 μL of freshly prepared TMB substrate solution was added. Color was determined 10 min after the addition of substrate at 650 nm by using a V_{max} reader. The dose–response curve was prepared by plotting the absorbance against the log of imazaquin concentration. The SoftMax program provided linear equation fits for the dose–response. On the basis of the equation, unknown values were quantified.

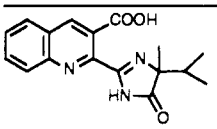
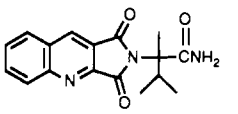
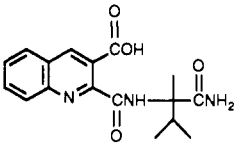
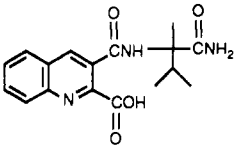
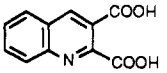
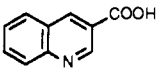
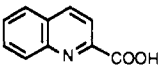
Specificity Determination. To determine the cross-reactivity of the antibody, solutions of imazethapyr, imazapyr, and imazamethabenz methyl between 100 and 1.6 ng/mL in PBS were used in lieu of the imazaquin solution for a dose–response curve as described above. Equations based on the absorbance and log concentration of the competing compounds were generated.

Imazaquin metabolites and other structurally unrelated agrochemicals as listed in Tables I and II were also tested for cross-reactivity. For these compounds, solutions of concentrations at 1 mg/mL in acetone or methanol were prepared. Further dilutions in PBS were prepared for the assay. The concentration of a compound which produced a 50% reduction in absorbance caused by the enzyme conjugate binding to a known amount of antibody is defined as the IC_{50} value (50% inhibition concentration).

RESULTS AND DISCUSSION

Hapten and Immunogen Synthesis. To render any of the imidazolinone compounds immunogenic for antibody production, it was necessary to conjugate them to a large immunogenic protein. The free carboxyl group in most of the imidazolinones seemed to be a natural candidate for conjugation. However, since the biological activity of the herbicides requires the free carboxyl function and imidazolinone ring, we designed the hapten compound I with a reactive aldehyde function distal to the group of biological interest for conjugation. Conjugation through this aldehyde function allowed the maximal freedom of the imidazolinone features available to stimulate immune responses. The binding of the antiserum to [^{14}C]imazaquin (data not shown) was indicative of the successful design of the hapten molecule.

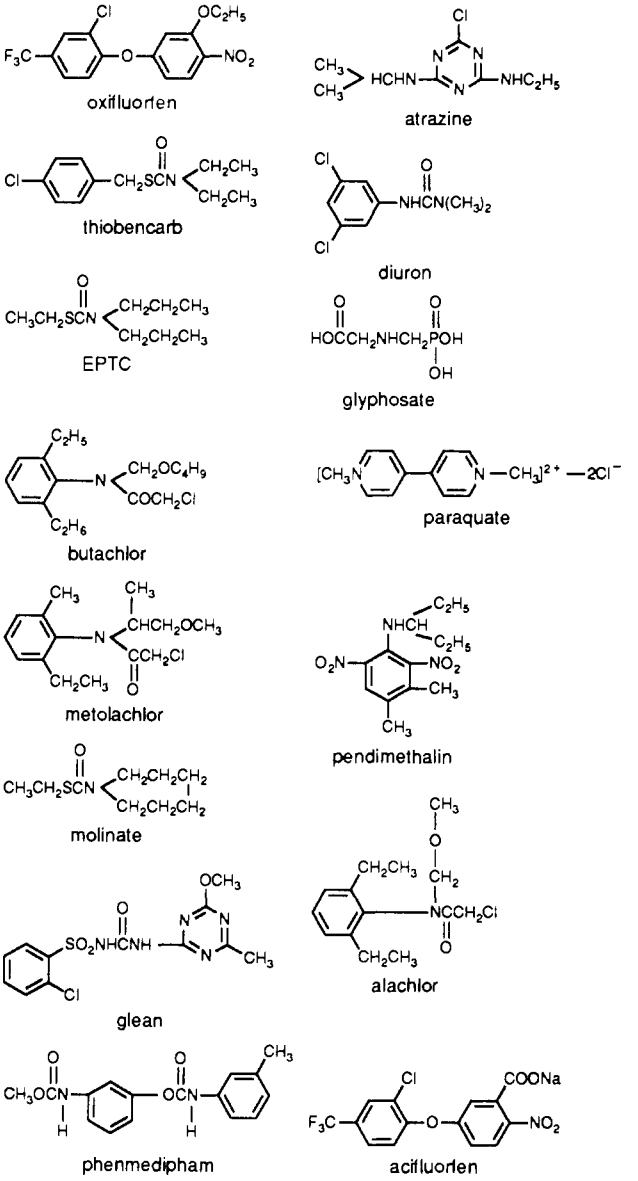
Table I. Cross-Reactivity of Imazaquin Metabolites

structure	chemical name	IC_{50}, ng/mL
	imazaquin	10.7
	1,3-dihydro-α-isopropyl-α-methyl-1,3-dioxo-2*H*-pyrrolo-[3,4-*b*]quinolin-2-acetamide	12 000
	2-[(1-carbamoyl-1,2-dimethyl-propyl)-carbamoyl]-3-quinolinecarboxylic acid	6 200
	3-[(1-carbamoyl-1,2-di-methylpropyl)-carbamoyl]-quinaldic acid	6 200
	2-carbamoyl-3-quinoline-carboxylic acid	>50 000
	3-quinolinecarboxylic acid	>50 000
	quinaldic acid	>50 000

Competitive Enzyme Immunoassay. We chose to use protein A as a bridge for coating antibody on a solid phase in the competitive assay system. Since protein A selectively binds to the Fc portions of immunoglobulins, the protein A bridge not only acts as a purification step for the serum but also helps to orient the antibodies for optimal antigen binding. Although not all immunoglobulin subclasses bind to protein A, those which did bind were able to provide a better precision in the assay. The titer of the best serum as determined by HRP–imazaquin binding through a protein A bridge was greater than 3000-fold dilution in this system. The linearity fit of the dose–response was usually better than 0.90 between the concentrations of 0.4 and 25 ng/mL.

Dose–Response Curves Using HRP–Compound I or HRP–Imazaquin. To further illustrate the need of having a relevant hapten–enzyme conjugate for a direct competitive immunoassay, we prepared HRP–compound I and HRP–imazaquin. The dose–response curves of direct competition between HRP–compound I and free imazaquin or HRP–imazaquin and free imazaquin for a constant amount of solid bound antibody were obtained (Figure 5). A dramatic difference between the curves was observed. With HRP–imazaquin, the displacement of enzyme conjugate by free imazaquin was steep due to the similarity in structure between the HRP–imazaquin and free imazaquin. However, when a benzene ring was substituted for the quinoline ring, the antibody fit was dramatically changed. The dose–response curve became flat, and the concentration of imazaquin spanned several orders of magnitude. In addition to the differences in structure between imazaquin and compound I, it is also likely that the imidazolinone ring of compound I has been reduced upon cyanoborohydride treatment during conjugation to the enzyme HRP. This increased the structural differences between the analyte imazaquin and the enzyme conjugate.

Table II. Non-Imidazolinone Agrochemicals



It is evident that for imazaquin quantitation HRP–imazaquin is a better competing molecule than compound I.

Dose–Response with Other Imidazolinone Herbicides. Dose–response curves for imazapyr, imazethapyr, imazamethabenz methyl, and imazaquin were obtained using a constant amount of HRP–imazaquin and the same concentration of coated antibody (Figure 6). Because of the structural similarity, all compounds competed against HRP–imazaquin for the antibody sites. It is interesting to note that even though the slopes for imazethapyr and imazamethabenz methyl dose–responses are similar to that of imazaquin, the curves are somewhat displaced. This indicates that the antibody has greater affinity toward imazaquin than imazethapyr or imazamethabenz methyl. Additionally, the presence of a methyl ester moiety as in imazamethabenz methyl did not eliminate the competitiveness of the compound toward displacing HRP–imazaquin. This indicated that a charged carboxyl group is not necessary for antibody binding. These results demonstrate that the imidazoli-

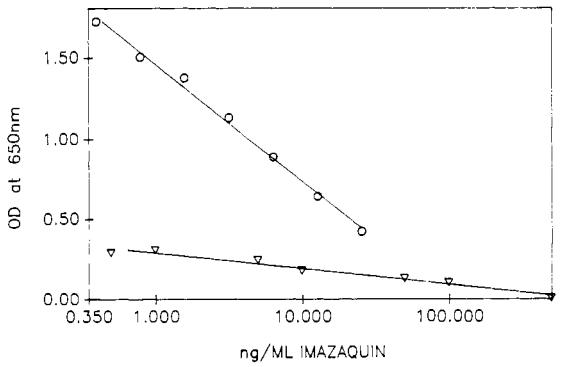


Figure 5. Comparison of dose–response curves using different HRP conjugates: (○) HRP–imazaquin; (▽) HRP–compound I. The log values of imazaquin concentration are plotted on the *X* axis, while the *Y* axis represents the enzyme activity as absorbance at 650 nm. Data represent the mean of triplicates with coefficient of variation below 6%.

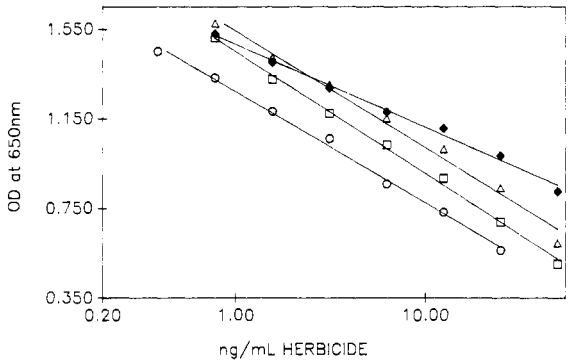


Figure 6. Dose–response curves of four imidazolinone herbicides. The assays used a constant amount of HRP–imazaquin and varying concentrations of herbicides: (○) imazaquin; (♦) imazapyr; (△) imazamethabenz methyl; (□) imazethapyr. The log of concentration is on the *X* axis and absorbance at 650 nm on the *Y* axis. Data represent the mean of triplicates.

none ring is a major epitope, and in the absence of appropriate enzyme conjugates, HRP–imazaquin may be useful for estimating the class of structurally similar imidazolinone herbicides by competitive immunoassay. A recent publication by Newsome and Collins (1991) demonstrated an immunoassay system for imazamethabenz and imazamethabenz methyl. The hapten imazamethabenz was conjugated to proteins via the carboxyl group for antibody production. It would be interesting to determine if this antibody also cross-reacts with all of the imidazolinone herbicides.

Specificity of Imazaquin ELISA System. Some of the known plant metabolites of imazaquin (Table I) were tested for their ability to displace HRP–imazaquin in the competitive ELISA. As shown in Figure 7, the metabolites can be classified into two groups: one with IC_{50} about 6000–12 000 ng/mL and the other without any response at concentrations as high as 50 000 ng/mL. Since the antibody was derived from a polyclonal serum, a small population of the antibody could recognize the partially degraded imazaquin, as evidenced by the group of compounds which exhibit IC_{50} values of 6000–12 000 ng/mL. However, it is evident that the metabolites do not interfere with the imazaquin ELISA in any significant manner. Attempts to determine IC_{50} values for several common agrochemicals (Table II) were made. There is no displacement of HRP–imazaquin binding at concentrations of 100 μg/mL. Higher concentrations were not tested due to the lack of solubility of the compounds in aqueous solution. Since concentrations of 100 μg/mL could not show displacement to the antibody–HRP imazaquin

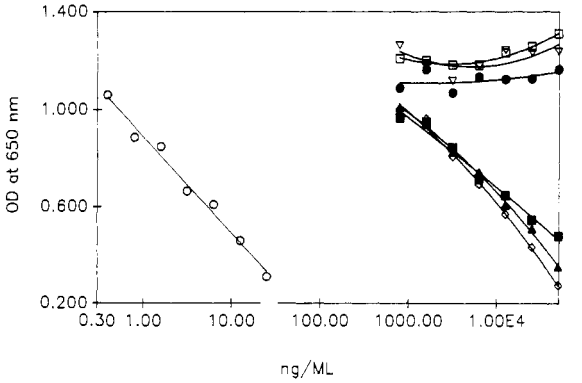


Figure 7. Dose–response curves of imazaquin metabolites compared with imazaquin: (○) imazaquin; (■) 3-[1-carbamoyl-1,2-dimethylpropyl)carbamoyl]quinaldic acid; (▲) 1,3-dihydro-α-isopropyl-α-methyl-1,3-dioxo-2*H*-pyrrolo[3,4-*b*]quinolin-2-acetamide; (△) 2-[(1-carbamoyl-1,2-dimethylpropyl)carbamoyl]-3-quinolinecarboxylic acid; (●) quinaldic acid; (□) 2-carbamoyl-3-quinolinecarboxylic acid; (▽) 3-quinolinecarboxylic acid.

binding, this immunoassay system is very specific for the class of imidazolinone compounds.

Conclusion. We have reported the synthesis of an immunogen useful for eliciting antibody against a group of imidazolinone herbicides. The direct competition assays employing HRP conjugates of compound I or imazaquin analogue showed that HRP–imazaquin was a better competing molecule for imazaquin quantitation. The dose–response curve for imazaquin ranged from 0.4 to 25 ng/mL. Dose–response curves for imazapyr, imazethapyr, or imazamethabenz methyl were obtained using this assay system. However, due to the minor differences between the HRP–imazaquin conjugate and these free analytes, the sensitivity of the dose–responses was slightly compromised. The specificity of the assay system was shown to be generic for the imidazolinone class of compounds, but no cross-reactivity was observed with all non-imidazolinone-containing agrochemicals tested.

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Registry No. I, 139493-37-1; methyl α-hydroxy-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*m*-toluate, 97712-15-7; imidazaquin, 81335-37-7; imidazapyr, 81334-34-1; imazethapyr, 81335-77-5; imazamethabenz methyl, 81405-85-8.