A General Method for Developing Immunoassays to Chloroacetanilide Herbicides

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Metolachlor, amidochlor, and butachlor were each conjugated to proteins via a thioether linkage. The molar ratios of haptens to protein ranged from 11 to 21 as determined by radioactivity. Polyclonal antibodies to metolachlor, amidochlor, and butachlor were obtained by immunizing rabbits with the corresponding hapten–protein conjugates. These antibodies were used in the development of sensitive inhibition ELISAs with I_{50} levels of less than 10 ppb. The individual ELISAs were highly specific, showing little or no cross-reactivity to other structurally similar chloroacetanilide herbicides.

INTRODUCTION

Immunoassays offer an alternative to conventional instrumental assays for the detection of herbicide residues. Immunoassays are cost-effective and sensitive and have a high throughput. These characteristics can be very advantageous over conventional instrumental assays in some applications (Hammock et al., 1990; Jung et al., 1989; Wratten and Feng, 1990a).

Alachlor and metolachlor (Figure 1) represent two of the most widely used of chloroacetanilide herbicides. We have previously reported on the development of an enzyme-linked immunosorbent assay (ELISA) for alachlor (Feng et al., 1990a,b). More recently, the development of an ELISA for metolachlor was reported (Schlaeppi et al., 1991).

Antibodies against alachlor were generated by use of an alachlor–protein conjugate (Feng et al., 1990a,b). Alachlor was conjugated to the protein carrier through the chlorine-bearing carbon via a thioether bond. This type of linkage was easily made based on the reactivity of the chlorine-bearing carbon with nucleophiles such as glutathione (Feng and Patanella, 1988). Furthermore, the sulfur atom in the conjugate, which is similar in size to the chlorine atom, facilitated the formation of antibodies that recognized alachlor. Conjugation of alachlor to proteins via the thioether linkage is a simple one-step reaction requiring no synthesis of novel haptens. Rabbit polyclonal antibodies generated using the thioether conjugate were useful in the development of a sensitive alachlor ELISA (I_{50} of 1 ppb). The ELISA discriminated alachlor from other chloroacetanilide herbicides, although strong cross-reactivity was detected with some thioether metabolites. The alachlor ELISA performed well in the analysis of environmental water samples (Feng et al., 1990a). Subsequently, an analogue of alachlor was conjugated to proteins through a carboxyl group in the methoxymethyl side chain (Sharp et al., 1991). Antibodies from the carboxy–alachlor conjugate showed even greater specificity toward alachlor and discriminated against other chloroacetanilides as well as alachlor thioether metabolites.

Using the same thioether conjugation method, we now report the development of ELISAs for three other chloroacetanilide herbicides: metolachlor, amidochlor, and butachlor (Figure 1). Our results demonstrate that these chloroacetanilide herbicides were easily conjugated to proteins via a thioether linkage, and the resulting hapten–protein conjugate elicited antibody production in rabbits.

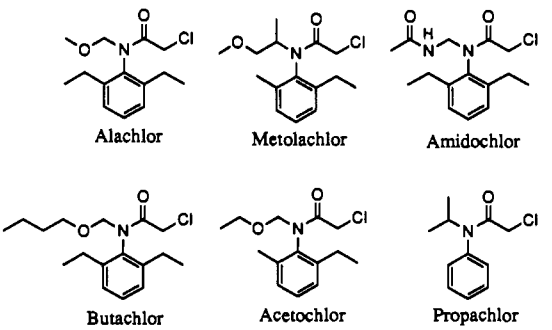

Figure 1. Structures of common chloroacetanilide herbicides.

The antibodies were useful in the development of inhibition ELISAs with low parts per billion levels of sensitivity.

MATERIALS AND METHODS

Materials. Human serum albumin (HSA), bovine serum albumin (BSA), sheep γ-immunoglobulin (IgG), and *o*-phenylenediamine (PDA) were purchased from Sigma Chemical Co. *S*-Acetylmercaptosuccinic anhydride (AMSA) and *N*-acetylhomocysteine thiolactone (AHT) were obtained from Aldrich Chemical Co. Immulon 1 96-well microtiter plates were purchased from Dynatech. Goat anti-rabbit γ-globulin conjugated to horseradish peroxidase (GAR–HRP) was obtained from Cooper Biomedical Co. Freund's complete and incomplete adjuvants were obtained from Difco Laboratories. Nonfat dry milk powder (Food Club brand), obtained locally, was used for blocking.

Uniformly [^{14}C]phenyl-labeled metolachlor was purchased from Pathfinder Laboratories. Uniformly [^{14}C]phenyl-labeled amidochlor and butachlor were provided by Dr. Robert Freeman (Monsanto Agricultural Co.). Radiolabeled haptens were diluted to approximately 0.06 mCi/mmol for conjugation to proteins.

Instrumentation. Spectrophotometric absorbances of 96-well microtiter plates were recorded on a Bio-Tek El 310 reader equipped with a 490-nm filter. The plates were washed with a Dynatech Dynawasher II. A 12-channel Titertek pipet (50–200 μL) from Flow Laboratories was used for dispensing liquids.

Synthesis of Hapten–Protein Conjugates. Radiolabeled haptens (metolachlor, amidochlor, and butachlor) were attached to carrier proteins (HSA or IgG) according to a previously described procedure (Feng et al., 1990a). Each of the haptens (25 molar equiv) was conjugated to HSA (1 molar equiv) using AHT (25 molar equiv) as the linker. Similarly, each of the haptens (25 molar equiv) was conjugated to sheep IgG (1 molar equiv) using AMSA (25 molar equiv) as the linker. The hapten–protein conjugates were purified by dialysis, lyophilized, and stored at −20 °C. The conjugation ratio of hapten to protein was calculated on the basis of radioactivity as determined by liquid scintillation

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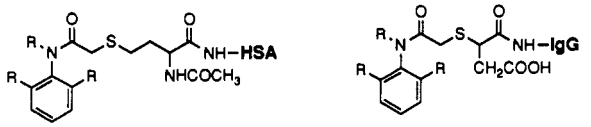

Hapten-AHT-HSA Hapten-AMSA-IgG

Figure 2. Structures of hapten–protein conjugates. Chloroacetanilides were conjugated to human serum albumin (HSA) or sheep immunoglobulin G (IgG) using *S*-acetylmercaptosuccinic anhydride (AMSA) or *N*-acetylhomocysteine thiolactone (AHT).

counting. The hapten–AMSA–IgG was used as the immunogen, and the hapten–AHT–HSA was used as the coating antigen in ELISAs.

Antibody Generation. Each of the hapten–AMSA–IgG conjugates (1 mg) in Freund's complete adjuvant was injected intradermally into three New Zealand white rabbits. Animals were boosted at 3–4-week cycles with 0.1–0.5 mg of the same hapten–protein conjugate in Freund's incomplete adjuvant. Animals were bled 7–10 days after each boost. Antisera were aliquoted and stored at −80 °C.

Immunoassays and Cross-Reactivity Studies. A checkerboard assay (Campbell, 1984) was conducted with each of the bleeds from different animals. The checkerboard assay selected the combination of antiserum dilution and coating antigen concentration (hapten–AHT–HSA) that would provide the greatest sensitivity in ELISAs. The optimized ELISA for metolachlor, amidochlor, and butachlor employed a coating antigen concentration between 5 and 50 ng/well and an antiserum dilution between 3000 and 5000.

The procedure for the inhibition ELISA was described previously (Feng et al., 1990a). PDA was used as the substrate for horseradish peroxidase; the reaction produced a chromophore which was detected at 490 nm. Standards were prepared in deionized water. Each sample was analyzed in six replicate wells. The median absorbance was then divided by the median absorbance from control wells (no coating antigen) to obtain the percentage of absorbance.

The optimized ELISAs for metolachlor, amidochlor, and butachlor were used to examine the cross-reactivity of the antibodies for six chloroacetanilides: alachlor, metolachlor, amidochlor, butachlor, acetochlor, and propachlor. Analytical grade samples of each analyte were dissolved in deionized water (10 and 50 ppb) and used to examine the antibody cross-reactivities of the ELISAs. With the inhibition ELISA format, analytes that do not react with the antibodies would produce absorbances near 100%; conversely, analytes that do react with the antibodies would produce a reduction in percentage of absorbance.

RESULTS AND DISCUSSION

Synthesis of Hapten–Protein Conjugates. Conjugation of chloroacetanilide herbicides to proteins is easily accomplished in a one-step reaction via a thioether linkage (Feng et al., 1990a). Metolachlor, amidochlor, and butachlor were conjugated to HSA using AHT and to sheep IgG using AMSA (Figure 2). AHT (Singer et al., 1960) and AMSA (Klotz and Heiney, 1962) are thiolating agents that react with the ϵ-amino group of lysine residues in proteins. The resulting thiols then displace the chlorine atom in haptens to produce the thioether linkage. By using different linkers and protein carriers, we eliminated the detection of antibodies generated against the linker and/or the carrier.

The use of radiolabeled haptens facilitated the determination of hapten to protein conjugation ratios. We feel that a hapten to protein molar ratio of 10–20 is optimal for the generation of antibodies. On the basis of radioactivity, the hapten to protein ratios for metolachlor, amidochlor, and butachlor were calculated (Table I). The molar ratio of hapten to HSA conjugates ranged between 11 and 14, and the molar ratio of hapten to IgG conjugates ranged between 12 and 21. These conjugation ratios were similar to what was obtained previously with alachlor (Feng et al., 1990a). In that experiment, the conjugation ratio for alachlor–AHT–BSA and alachlor–AMSA–IgG was measured at 12 and 19, respectively.

Table I. Conjugation Ratios of Haptens to Proteins (Moles/Mole) As Determined by Radioactivity Analysis

coating antigen	molar ratio, hapten/protein	immunogen	molar ratio, hapten/protein
metolachlor–AHT–HSA	11	metolachlor–AMSA–IgG	15
amidochlor–AHT–HSA	13	amidochlor–AMSA–IgG	12
butachlor–ATH–HSA	14	butachlor–AMSA–IgG	21

Checkerboard Assay and ELISA. Each of the IgG conjugates was immunized into three rabbits. The antisera were aliquoted and stored at −80 °C and were freshly thawed for each experiment. A checkerboard assay (Campbell, 1984) was used to screen varying concentrations of the antiserum with varying concentrations of the coating antigen (hapten–AHT–HSA). The checkerboard assay detected the presence of antibodies that bound the coating antigen. Bleeds from each of the rabbits were screened by checkerboard assays. For each hapten–protein conjugate, at least two of the three rabbits produced antibodies that recognized the coating antigen. Animals were boosted for up to six cycles to maximize the antibody titer.

The results from the checkerboard assays provided the optimal combination of antiserum dilution and coating antigen concentration for the ELISAs. The antisera were judged in terms of titer, sensitivity, and background. The best antiserum from a rabbit was then selected for further development of the inhibition ELISA. In this type of assay, the reactivity of the antibodies with the coating antigen is inhibited by the presence of free hapten. Increasing the hapten concentration decreased the level of bound antibody and lowered the percentage of absorbance.

Metolachlor ELISA. Two of the three rabbits immunized with metolachlor–AMSA–IgG produced antibodies against metolachlor. The optimized ELISA for metolachlor employed a coating antigen (metolachlor–AHT–HSA) level of 40 ng/well and a serum dilution of 3500. The I_{50} for metolachlor was estimated at 6 ppb.

Amidochlor ELISA. All three rabbits immunized with amidochlor–AMSA–IgG produced antibodies against amidochlor. The optimized ELISA for amidochlor employed a coating antigen (amidochlor–AHT–HSA) level of 20 ng/well and a serum dilution of 3500. The I_{50} for amidochlor was estimated at 10 ppb.

Butachlor ELISA. Two of three rabbits immunized with butachlor–AMSA–IgG produced antibodies against butachlor. The optimized ELISA for butachlor employed a coating antigen (butachlor–AHT–HSA) level of 50 ng/well and a serum dilution of 2000. The I_{50} for butachlor was estimated at 7 ppb.

Cross-Reactivity Studies. The inhibition ELISAs demonstrated that the individual antibodies were able to react with the corresponding free hapten. The specificity of the individual antibodies was examined againt six commonly used commercial chloroacetanilide herbicides: alachlor, metolachlor, amidochlor, butachlor, acetochlor, and propachlor. Standard solutions (10 and 50 ppb) of the six herbicides in water were analyzed by ELISAs for metolachlor, amidochlor, and butachlor. Herbicides that showed cross-reactivity with the antibodies resulted in lower percentages of absorbance (Figure 3).

When tested against standard solutions (10 and 50 ppb) of the six herbicides, the metolachlor ELISA showed a concentration-dependent reduction in percentage of ab-

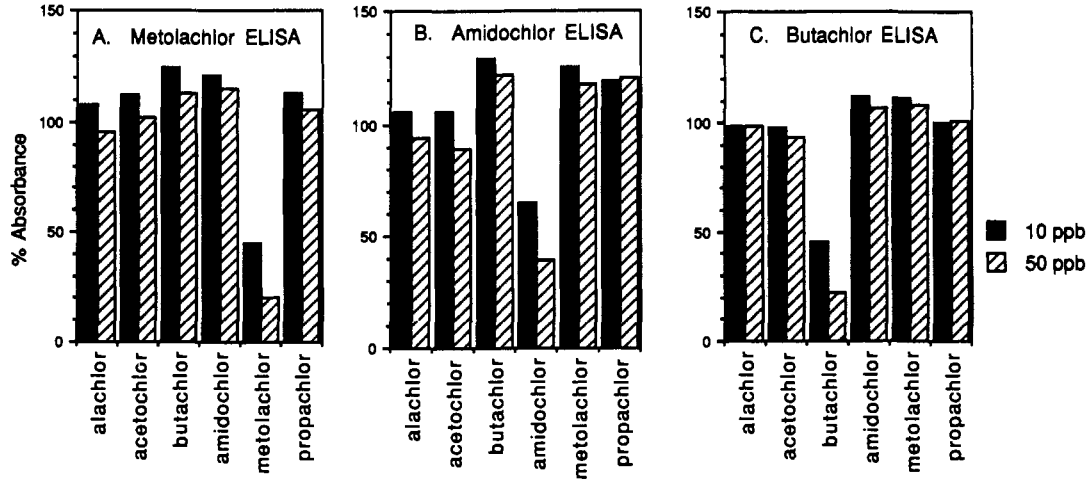

Figure 3. Specificity of ELISAs for chloroacetanilide herbicides. ELISAs were conducted with 10 and 50 ppb of each analyte. Cross-reactivities of antibodies for the analytes produced a reduction in percentage of absorbance.

sorbance only in the presence of free metolachlor (Figure 3A). The presence of the other five herbicides, at a level of up to 50 ppb, showed no significant reduction in percentage of absorbance. The metolachlor antibodies appeared to show little cross-reactivity to the other structurally related chloroacetanilides. Similar results were observed with the amidochlor ELISA (Figure 3B) and the butachlor ELISA (Figure 3C). These results demonstrate that the antibodies raised with the specific hapten–protein conjugate are only specific to the corresponding free hapten and show no significant cross-reactivity to other structurally similar chloroacetanilides. These results were not unexpected, since a similar pattern of cross-reactivity was observed previously with the alachlor ELISA (Feng et al., 1990a). We believe that the remarkable specificity of these antibodies is mainly attributed to the structure of hapten–protein conjugates. The major structural feature that distinguishes one chloroacetanilide from the other is the *N*-alkyl side chain. The thioether conjugation preserves this unique feature; consequently, the antibodies show great discrimination against other chloroacetanilides.

With the alachlor ELISA, the antibodies did show high cross-reactivity to several thioether metabolites of alachlor (Feng et al., 1990a). This was not surprising, in light of the structural similarity of the thioether metabolites to the alachlor–protein conjugate. Thioether metabolites of alachlor have been reported as products of animal metabolism (Sharp, 1988). The absence of thioether metabolites in environmental samples has permitted us to successfully apply the alachlor ELISA in the analysis of environmental water samples (Feng et al., 1990a). Presumably the ELISAs for metolachlor, amidochlor, and butachlor would also show a high cross-reactivity for their corresponding thioether metabolites.

Improving the Sensitivity of Amidochlor ELISA. Further optimization of ELISA for amidochlor showed an I_{50} of approximately 2 ppb (Figure 4). This assay employed 20 ng/well of the coating antigen (amidochlor–AHT–HSA). The sensitivity of any inhibition ELISA is affected by the strength of interaction between the antibody and the coating antigen. A stronger interaction requires a greater concentration of the free hapten to inhibit that reaction, thereby reducing the sensitivity of the assay. Therefore, one way to increase the sensitivity of the assay is to use a different coating antigen that shows less interaction with the antibodies.

Checkerboard assays were conducted with the amidochlor antisera using three different coating antigens: alachlor–AHT–BSA, metolachlor–AHT–HSA, and butachlor–AHT–HSA. Because of structural differences, these coating antigens are expected to show less interaction with the amidochlor antibodies. The optimized ELISAs required a wide range of coating antigen levels: 40 ng/well of alachlor–AHT–BSA, 640 ng/well of metolachlor–AHT–HSA, or 150 ng/well of butachlor–AHT–HSA. The optimized ELISAs were then used to analyze amidochlor standards in water (0.25–32 ppb). The data in Figure 4 show that the use of other coating antigens did improve the sensitivity of the amidochlor ELISA. The best assay employed butachlor–AHT–HSA as the coating antigen and produced an I_{50} of less than 0.2 ppb, which is a 10-fold improvement in sensitivity from the original assay employing amidochlor–AHT–HSA as the coating antigen. Presumably a similar approach could be used to improve the sensitivity of other ELISAs.

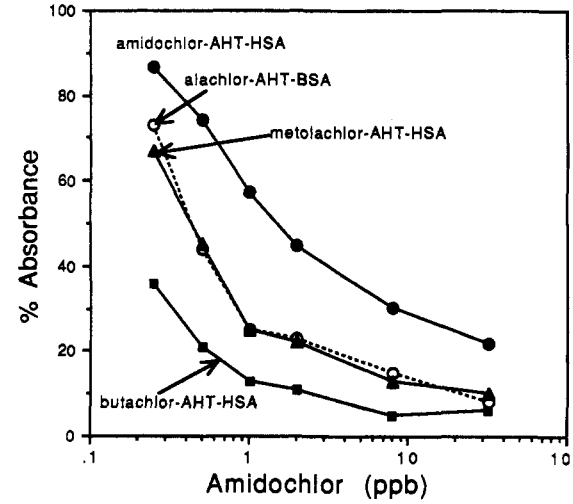

Figure 4. Effect of different coating antigens on the sensitivity of the inhibition ELISA for amidochlor. The coating antigen levels were amidochlor–AHT–HSA at 20 ng/well, alachlor–AHT–BSA at 40 ng/well, metolachlor–AHT–HSA at 640 ng/well, or butachlor–AHT–HSA at 150 ng/well.

Chloroacetanilides conjugated to proteins via the thioether linkage have successfully produced antibodies in four of four cases (alachlor, metolachlor, amidochlor, and butachlor). The resulting antibodies are highly discriminatory against other chloroacetanilides. The major limitation of these antibodies is their cross-reactivity to some thioether metabolites. Nevertheless, knowing the limitation of these antibodies has allowed us to find suitable

applications for these ELISAs. Validation studies will ultimately determine the successful application of these ELISAs for pesticide residue analysis.

Antibodies against alachlor and metolachlor have also been developed using other hapten–protein conjugation methods. An analogue of alachlor with a carboxyl functional group in the *N*-methoxymethyl side chain was successfully used as a hapten to generate polyclonal antibodies (Sharp et al., 1991). A more recent paper described the generation of monoclonal antibodies against metolachlor using a hapten containing a carboxybutoxy functional group in the phenyl ring (Schlaeppi et al., 1991). We believe that conjugation of chloroacetanilides to proteins via the thioether linkage, as described in the current paper, offers several advantages. Our data suggest that the thioether conjugation method is applicable to all chloroacetanilide herbicides. But more importantly, this method is easily carried out in a one-step reaction using the herbicide itself, thus eliminating the need for hapten synthesis.

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LITERATURE CITED

Campbell, A. M. *Monoclonal Antibody Technology: The Production and Characterization of Rodent and Human Hybridomas*; Burdon, R. H., Knippenberg, P. H., Eds.; Elsevier: New York, 1984.

Feng, P. C. C.; Patanella, J. E. Identification of Mercapturic Acid Pathway Metabolites of Alachlor Formed by Liver and Kidney Homogenates of Rats, Mice, and Monkeys. *Pestic. Biochem. Physiol.* **1988**, *31*, 84–90.

Feng, P. C. C.; Wratten, S. J.; Horton, S. R.; Sharp, C. R.; Logusch, E. W. Development of an Enzyme-Linked Immunosorbent Assay for Alachlor and Its Application to the Analysis of Environmental Water Samples. *J. Agric. Food Chem.* **1990a**, *38*, 159–163.

Feng, P. C. C.; Wratten, S. J.; Logusch, E. W.; Horton, S. R.; Sharp, C. R. Immunoassay Detection Methods for Alachlor: Application to Analysis of Environmental Water Samples. In *Immunochemical Methods for Environmental Analysis*; Van Emon, J. M., Mumma, R. O., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990b; pp 180–192.

Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q. X.; Lucas, A. D.; Szekacs, A.; Sundaran, K. M. S. Immunochemical Technology in Environmental Analysis: Addressing Critical Problems. In *Immunochemical Methods for Environmental Analysis*; Van Emon, J. M., Mumma, R. O., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; pp 112–139.

Jung. F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X; Hammock, B. D. Use of Immunochemical Techniques for the Analysis of Pesticides. *Pestic. Sci.* **1989**, *26*, 303–317.

Klotz, I. M.; Heiney, R. E. Introduction of Sulfhydryl Groups into Proteins Using Acetylmercaptosuccinic Anydride. *Arch. Biochem. Biophys.* **1962**, *96*, 605–612.

Schlaeppi, J. M.; Moser, H.; Ramsteiner, K. Determination of Metolachlor by Competitive Enzyme Immunoassay Using a Specific Monoclonal Antibody. *J. Agric. Food Chem.* **1991**, *39*, 1533–1536.

Sharp, C. R.; Feng, P. C. C.; Horton, S. R.; Logusch, E. W. Development of Highly Specific Antibodies to Alachlor by Use of a Carboxy-Alachlor Protein Conjugate. In *Pesticide Residues and Food Safety*; Tweedy, B. G., Dishburger, H. J., Ballantine, L. G., McCarthy, J., Eds.; ACS Symposium Series 446; American Chemical Society: Washington, DC, 1991; pp 87–95.

Sharp, D. B. Alachlor. In *Herbicides: Chemistry, Degradation and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1988; pp 301–333.

Singer, S. J.; Fothergill, J. E.; Shainoff, J. A. General Method for the Isolation of Antibodies. *J. Am. Chem. Soc.* **1960**, *82*, 565–571.

Wratten, S. J.; Feng, P. C. C. Pesticide Analysis by Immunoassays. In *Development and Applications of Immuno-assays for Food Analysis*; Rittenburg, J. H., Ed.; Elsevier: New York, 1990; pp 201–220.

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