Hapten Design in the Development of Competitive Enyme-Linked Immunosorbent Assays for Genotoxic Metabolites of Alachlor

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The acetanilide compounds 2-chloro-2′,6′-diethylacetanilide (CDA) and 2-hydroxy-2′,6′-diethylacetanilide (HDA) are environmental degradative products of the chloroacetanilide herbicide alachlor. CDA, HDA, and alachlor are ground and surface water contaminants. CDA and HDA are genotoxic in bacterial and mammalian test systems. This paper reports hapten design in the development of two competitive enzyme-linked immunosorbent assays (cELISA) for the detection of CDA and HDA. Chloroacetanilide herbicides and other alachlor metabolites that may be present in environmental samples do not cross-react with the detection of CDA and HDA. Solid-phase extraction of CDA and HDA residues from aqueous samples results in a 1000-fold concentration factor, resulting in an effective detection limit of 15 pg/mL for both assays. The specificity of the cELISAs required preservation of the degree of substitution of the acetanilide moiety in the hapten design. The hapten synthesis strategies are suitable for metabolites of other chloroacetanilide herbicides (i.e., acetochlor, butachlor, metolachlor, and propachlor).

Keywords: *2-Chloro-2′,6′-diethylacetanilide; 2-hydroxy-2′,6′-diethylacetanilide; alachlor; chloroacetanilide herbicides; metabolites; cELISA*

INTRODUCTION

The study of the chemistry, fate, transport, and analysis of environmental metabolites of pesticides has historically not received as much attention as research on the parent pesticides themselves. This has been due to the large number of potential analytes arising from the breakdown of any given parent compound, coupled with a backlog of environmental research still needed for existing and newly developed pesticides. Degradation in the environment, whether biotic, abiotic, or photolytic, most commonly results in compounds that retain the structural core of the parent pesticide. These degradation products may also retain some of the activity of the parent and/or exhibit mechanisms of toxicity unrelated to the mode of action of the parent (Barrett, 1996). Metabolites are typically more polar than the parent compound and can be stable and therefore more mobile in the environment. In this manner, metabolites may have an even greater impact on ground and surface waters than the parent compound. Except for the sulfoxide and sulfone metabolites of aldicarb, there are no established tolerances for pesticide metabolites in water (Barbash and Resek, 1996).

The biotic, primarily microbial, degradation of alachlor results in a variety of aromatic amine metabolites that also have been detected in ground and surface waters (Potter and Carpenter, 1995; Pereira and Hostettler, 1993, Pereira et al., 1992; Pereira and Rostad, 1990; Aizawa, 1982; Tiedje and Hagedorn, 1975). Abiotic oxidative mechanisms play only a minimal role in degradation, and mineralization does not occur (Novick, 1986). Thus, movement of these metabolites out of the microbially active zone of the soil profile (i.e., the rhyzosphere) may result in some of these metabolites existing as long-term or terminal residues with the potential to contaminate ground and surface waters.

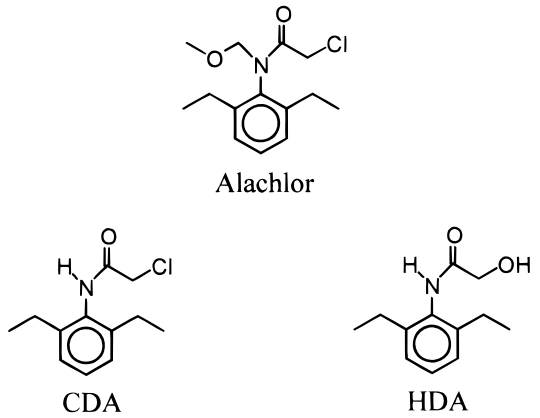

Figure 1. Chloroacetanilide herbicide alachlor and environmental degradative products CDA and HDA.

Alachlor (Figure 1) has been detected in the rhyzosphere for up to one year after application (Heyer and Stan, 1995), thus providing long-term opportunities for metabolite formation. Potter and Carpenter (1995) identified 20 distinct compounds in ground water samples that were postulated to arise from alachlor degradation. The major metabolites of alachlor that have been studied to date are alachlor−ethanesulfonic acid (alachlor-ESA; Thurman et al., 1996; Baker et al., 1993), 2-chloro-2′,6′-diethylacetanilide (CDA; Figure 1; Potter and Carpenter, 1995; Pereira and Hostettler, 1993; Pereira et al., 1992; Pereira and Rostad, 1990), and

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2-hydroxy-2′,6′-diethylacetanilide (HDA; Figure 1; Potter and Carpenter, 1995). These compounds exhibit environmental mobility and have been detected in both ground and surface waters at part per trillion levels. Nevertheless, the rate of formation, persistence, movement, and ultimate environmental fate for these metabolites of alachlor is unknown.

We have previously reported on the chronic toxicity potential of environmental metabolites of alachlor where it was determined that CDA and HDA are mutagenic in the *Salmonella*/microsome assay (Tessier and Clark, 1995). CDA has also been shown to form adducts with calf thymus DNA (Nelson and Ross, 1998) and increase the frequency of sister chromatid exchange in human lymphocytes (Hill et al., 1997). Therefore, the quantitation of the overall environmental impact of alachlor use in agriculture should include the added burden of ground and surface water contamination by the genotoxic metabolites CDA and HDA.

To date, the low-level detection of CDA and HDA in water samples has been accomplished by gas chromatography with ion-trap mass spectrometry (Pereira et al., 1990). Although this methodology allows determination of part per trillion level residues in water samples, the cost of instrumentation and the skill level required to operate it are prohibitive for significant, large-scale monitoring programs. The development of immunoassay technology specifically directed at the detection of CDA and HDA was deemed meritorious in that it would compliment chromatographic analysis of water and soil by providing the ability to screen large numbers of samples quickly and inexpensively. More involved analytical procedures can thus be performed as confirmation of a much smaller set of positive samples. This in turn would provide a better understanding of the environmental fate of CDA and HDA and whether operational factors, such as formulation type or soil management practices, mitigate or enhance their formation. Ultimately, this will clarify how the environmental degradation of alachlor occurs and which environmental factors result in the increased formation of mutagenic metabolites.

We have reported the development of a single competitive enzyme-linked immunosorbent assay (cELISA) that detects both CDA and HDA with approximately equal affinity (Tessier and Clark, 1998). Here we report on our studies of suitable hapten design for cELISAs that detect CDA and HDA individually. For the production of CDA and HDA specific antibodies, we synthesized four distinct hapten−carrier conjugates, utilizing three different attachment points on CDA (Figure 2). Preservation of the degree of substitution of nitrogen in the amide moiety (i.e., whether the hapten contained a secondary or tetiary amide) was critical for the ability of the resulting antisera to distinguish CDA and HDA (both secondary amides) from the parent compound alachlor (a tertiary amide). The resulting hapten design strategies are applicable to the development of immunoassays for metabolites of other chloroacetanilide herbicides.

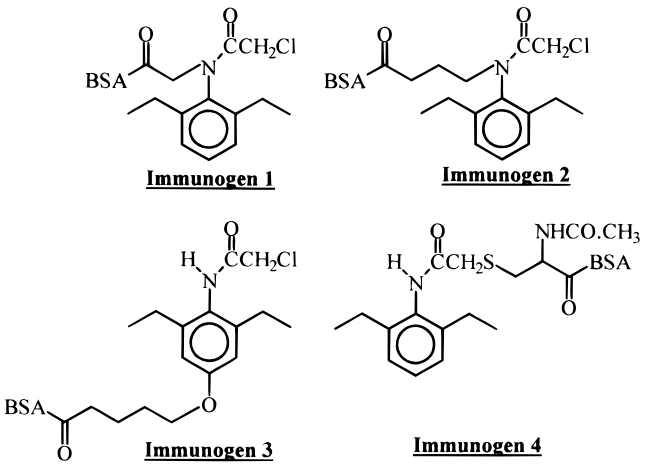

Figure 2. Immunogens synthesized for the production of polyclonal antisera against the target analytes. Note that immunogens 1 and 2 change the degree of substitution of the amide moiety in the target analyte (i.e., from a secondary to a tertiary amide). Immunogens 3 and 4 preserve the degree of substitution of the target analyte amide moiety.

EXPERIMENTAL PROCEDURES

Chemicals. 2,6-Diethylaniline, ethyl chloroacetate, chloroacetyl chloride, 4-ethylphenol, aluminum chloride, and sodium nitrite were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N*-Hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide, *N*-acetyl homocysteine thiolactone, ethyl 4-bromobutyrate, ethyl 5-bromovalerate, bovine serum albumin, human serum albumin, and ovalbumin were obtained from Sigma (St. Louis, MO). Goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). 2-Chloro-2′,6′-diethylacetanilide (99%) and 2-hydroxy-2′,6′-diethylacetanilide (99%) were synthesized in the Mass Spectrometry Laboratory, College of Food and Natural Resources, University of Massachusetts−Amherst (Potter and Carpenter, 1995).

Instrumentation. Infrared (IR) analyses were conducted on a Perkin-Elmer 1330 spectrophotometer (Norwalk, CT) scanning from 4000 to 200 cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) analyses were conducted on a Bruker/IBM 200AC (Karlsruhe, Germany) spectrometer operating at 200 MHz. Chemical shifts in parts per million are reported compared to a tetramethylsilane (TMS) standard. Elemental analyses were conducted by the Microanalytical Laboratory, Department of Chemistry, University of Massachusetts−Amherst. Gas chromatographic/mass spectral (GC/MS) analyses were conducted on a Hewlett-Packard 5890 II gas chromatograph/5971 mass selective detector (Palo Alto, CA). The injector temperature was 250 °C and the interface 280 °C, and the mass range scanned was from 35 to 550 mass units. For hapten synthesis intermediate analysis, the chromatographic column was a 30 m HP-5 (Hewlett-Packard, Palo Alto, CA) column, 0.25 μm film, temperature programmed from 70 °C (1 min hold) to 275 °C (1 min hold) at 15 °C/min. Analysis of CDA and HDA in water sample extracts was performed on a 30 m DB-5, 0.25 μm film (J&W, Folsom, CA), programmed from 100 °C (1 min hold) to 250 °C (0.5 min hold) at 20 °C/min. Injection volume was 2 μL. Immunoassays were determined on a Softmax plate reader (Molecular Devices, Sunnyvale, CA) operating at 450 nm.

Synthesis of Haptens. *Hapten C2−CDA and Hapten C4−CDA.* The synthesis of haptenic derivatives of CDA containing either a two-carbon (C2-CDA) or a four-carbon (C4-CDA) spacer group attached to the nitrogen moiety for subsequent linkage to carrier protein molecules is outlined schematically in Figure 3.

*Ethyl 2-(2,6-diethyl)anilinoethanoate (**2**).* 2,6-Diethylaniline (7.5 g, 0.05 mol) and ethyl chloroacetate (12.3 g, 0.1 mol) were heated with stirring in the presence of Na_2CO_3 (4.2 g, 0.05 mol) for 4 h at 140 °C. The reaction mixture was removed from heat and dH_2O added. The crude reaction mixture was extracted with ethyl acetate, which was then washed with 1 M HCl and dried over Na_2SO_4. Removal of solvent yielded 11.4 g of crude **2**. The crude product was purified by dry column chromatography [63−200 μm silica, 40% (v/v) ethyl acetate/

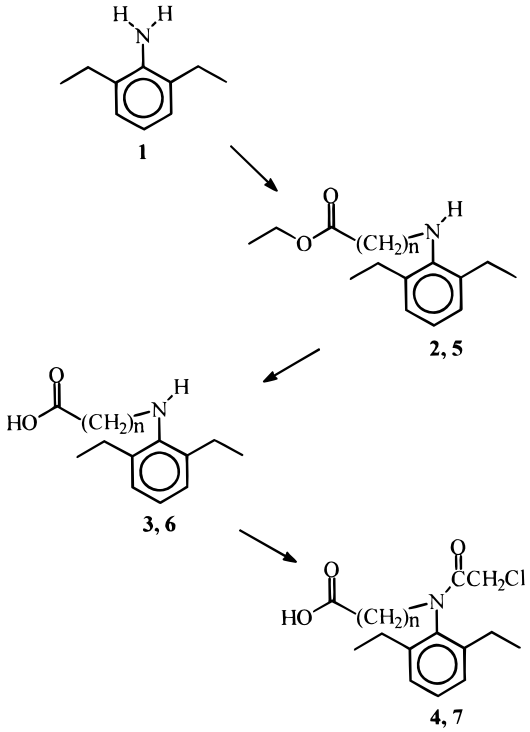

Figure 3. Synthesis of haptenic derivatives of CDA featuring either a two-carbon (compound **4**; $n = 1$) or a four-carbon (compound **7**; $n = 2$) spacer group attached to the nitrogen moiety.

hexane) to yield an amber oil (90%]: IR (neat, cm^{-1}) 3380 (NH), 1740 (C═O), 1370−1340 (2° aryl CN), 1205 (ester COC); ^1H NMR ($CDCl_3$) δ 1.2 (t, 9H, $-CH_3$), 2.7 (q, 4H, $-CH_2-$), 3.8 (s, 2H, NCH_2-), 4.0 (s, 1H, NH), 4.2 (q, 2H, $-COOCH_2-$), 7.0 (m, 3H, aryl −CH); GC/MS $t_r = 8.9$ min, m/z 235 (M^+), 162, 147.

*2-(2,6-Diethyl)anilinoethanoic Acid (**3**).* Ethyl 2-(2,6-diethyl)-anilinoethanoate (**2**, 1.0 g) was dissolved in 0.7 M NaOH/acetone (65:35 v/v). The reaction mixture was stirred at ambient temperature for 14 h. Acetone was removed under vacuum and the aqueous mixture acidified (H_3PO_4) to pH 3−4. Crude **3** was extracted with ethyl acetate. After drying (Na_2SO_4) and removal of solvent, the product was purified by dry column chromatography [63−200 μm silica, 25% (v/v) ethyl acetate/hexane] to yield an amber oil (80%): IR (neat, cm^{-1}) 3380 (NH), 3300−2500 (OH), 1730 (C═O), 1400−1380 (CN), 1220 (COOH); GC/MS (methyl ester) $t_r = 8.3$ min, m/z 221 (M^+), 162, 147, 132.

*2-[2-Chloro-(2′,6′-diethyl)acetanilido]ethanoic Acid (**4**).* 2-(2,6-Diethyl)anilinoethanoic acid (**3**, 1.5 g) was dissolved in methylene chloride. Chloroacetyl chloride (1 g) in methylene chloride was added *dropwise* with stirring (CAUTION! May react violently.) to the solution of **3**. The reaction continued for 2 h at ambient temperature with constant stirring. The reaction mixture was washed with dH_2O and dried (Na_2SO_4), and the solvent was removed to yield 1.4 g of a purple oil. The product was purified by dry column chromatography [63−200 μm silica, 25% (v/v) ethyl acetate/hexane] to yield an amber oil (84%): IR (neat, cm^{-1}) 3300−2500 (OH), 1735 (C═O), 1680 (C═O), 1430−1380 (CN), 1250−1200 (COOH); GC/MS (methyl ester) $t_r = 11.6$ min, m/z 297 (M^+), 248, 188, 160.

*Ethyl 2-(2,6-Diethyl)anilinobutanoate (**5**).* 2,6-Diethylaniline (**1**, 7.5 g, 0.05 mol) and ethyl 4-bromobutanoate (19.5 g, 0.1 mol) were reacted as for compound **2**. The crude product was purified by dry column chromatography [63−200 μm silica, 10% (v/v) ethyl acetate/hexane] to yield a pale yellow oil (90%): IR (neat, cm^{-1}) 1750 (C═O), 1360 (secondary aryl CN), 1250 (ester COC); GC/MS $t_r = 11.5$ min, m/z 263 (M^+), 218, 162, 148, 132.

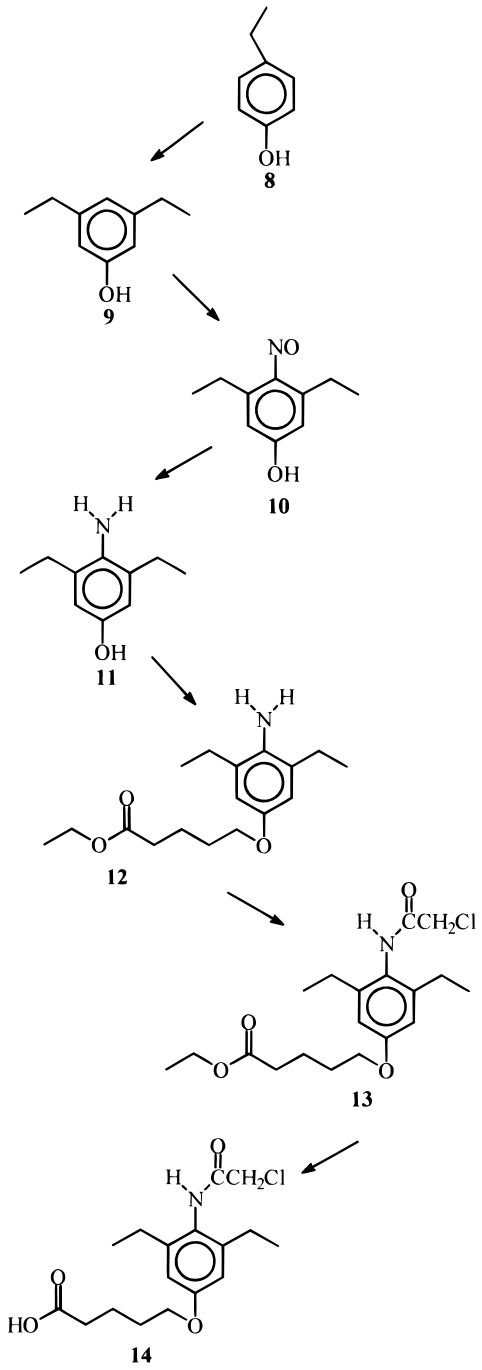

Figure 4. Synthesis of a haptenic derivative (compound **14**) of CDA featuring a five-carbon spacer group attached to the aromatic ring.

*2-(2,6-Diethyl)anilinobutanoic Acid (**6**).* Ethyl 2-(2,6-diethyl)-anilinobutanoate (**5**, 1.0 g) was hydrolyzed as for compound 3.

*2-(2-Chloro-(2′,6′-diethyl)acetanilido)butanoic Acid (**7**).* The chloroacetanilide moiety was attached to *2*-(2,6-diethyl)anilinobutanoic acid (**6**, 0.75 g) as for compound **4**.

Hapten Phe-CDA. The synthesis of a haptenic derivative of CDA containing a five-carbon spacer group attached to the aromatic ring (Phe-CDA) for subsequent linkage to carrier protein molecules is outlined schematically in Figure 4.

*3,5-Diethylphenol (**9**).* 4-Ethylphenol (**8**, 50 g, 0.41 mol) and aluminum chloride (125 g, 0.94 mol) were heated with stirring at 110 ± 5 °C for 4 h. The reaction mixture was cooled to 60 °C, poured onto crushed ice, and steam distilled to yield 41 g of a crude phenolic mixture. The phenolic mixture was fractionally distilled under vacuum (15 mmHg). 3,5-Diethyl-

phenol, collected as the remaining fraction after removal of phenol and related byproducts, was recrystallized from petroleum ether to yield 3.8 g of white, needlelike crystals with a distinct phenolic odor: mp 77 °C; purity, 99.9% (GC/MS); IR (KBr, cm^{-1}) 3320−3180 (s, b, OH), 1600, 1450 (aryl ring); GC/MS t_r = 10.27 min, *m*/*z* 150 (M^+), 135 (M − CH_3), 121 (M − C_2H_5).

*3,5-Diethyl-4-nitrosophenol (**10**).* 3,5-Diethylphenol (**9**; 3.6 g, 24 mmol) was dissolved in ethanol, and an equal volume of concentrated HCl was added with stirring. The reaction mixture was cooled to 0 °C, and $NaNO_3$ (2.5 g, 30 mmol), dissolved in dH_2O, was added dropwise. The mixture was stirred for 2 h at 10 °C and then poured onto ice−water and filtered to yield 3.4 g of rust-colored crystals: mp 126 °C (dec); IR (KBr, cm^{-1}) 1500 (s, N═O), 1140 (s, COH), 1000 (CN).

*4-Amino-3,5-diethylphenol (**11**).* 3,5-Diethyl-4-nitrosophenol (**10**; 3.4 g in THF) was hydrogenated at atmospheric pressure in the presence of palladium catalyst (380 mg). [CAUTION! The palladium catalyst can ignite solvent vapors. The hydrogenation apparatus must be flushed with inert gas prior to addition of catalyst.] The hydrogenation reaction continued for 3 h, after which time, the reaction solution was filtered through Celite 545. Removal of the solvent yielded 3 g of orange crystals: mp 118 °C; purity, 97.6% (GC/MS); GC/MS t_r = 8.4 min, *m*/*z* 165 (M^+), 150 (M − CH_3), 135. Elemental analysis (theoretical values in parentheses): C, 72.38% (72.73); H, 9.3% (9.1); N, 8.36% (8.5); O, 9.96% (9.7).

*Ethyl 5-(4-Amino-3,5-diethyl)phenoxypentanoate (**12**).* 4-Amino-3,5-diethylphenol (**11**; 1 g, 6 mmol) was dissolved in dimethyl sulfoxide. The stirred solution was cooled to 15 °C, and KOH (0.5 g, 9 mmol) added. The solution was returned to ambient temperature, and ethyl 5-bromopentanoate (1.9 g, 9 mmol) was slowly added over the course of 70 min, after which time, the reaction was stopped by pouring the solution into 4 M HCl at 0 °C. The acidic solution was extracted with diethyl ether and then basified with 2 M NaOH. The basic solution was extracted again with diethyl ether, and the combined ethereal extracts were washed with dH_2O and brine and dried over Na_2SO_4. The solvent was removed to yield 0.3 g of a brown oil: purity, 99.7% (GC/MS); IR (neat, cm^{-1}) 1725 (C═O, ester), 1590 (−NH); GC/MS t_r = 14.14 min, *m*/*z* 293 (M^+), 248, 165/164, 129, 101.

*Ethyl 5-(4-Chloroacetamido-3,5-diethyl)phenoxypentanoate (**13**).* Ethyl 5-(4-amino-3,5-diethyl)phenoxypentanoate (**12**; 0.3 g, 1 mmol) was dissolved in methylene chloride. Chloroacetyl chloride (170 mg, 1.5 mmol, diluted in methylene chloride) was added at room temperature to the stirred solution of **12** in 100 μL aliquots at 2 min intervals, and the reaction continued for 30 min after the last chloroacetyl chloride addition. The reaction mixture was washed with dH_2O and brine and dried (Na_2SO_4), and the solvent was removed to yield 0.33 g of a brown solid: purity, 99.9% (GC/MS); IR (Nujol, cm^{-1}) 3240, 2940, 1740 (s, C═O); GC/MS t_r = 15.00 min, *m*/*z* 369 (M^+), 129, 101 (M − $C_2H_5CO{\cdot}C_2H_5$). Elemental analysis (theoretical values in parentheses): C, 61.55% (61.7); H, 7.57% (7.63); N, 3.68% (3.79); Cl, 9.38% (9.58); O, 17.82 (17.30).

*5-(4-Chloroacetamido-3,5-diethyl)phenoxypentanoic Acid (**14**).* Ethyl 5-(4-chloroacetamido-3,5-diethyl)phenoxypentanoate (**13**; 163 mg, 0.4 mmol), dissolved in acetone, was stirred in the presence of 2 M KOH at ambient temperature for 24 h. The reaction solution was neutralized with 4 M HCl and extracted with diethyl ether. The combined extracts were washed with dH_2O and brine and dried over Na_2SO_4. The solvent was removed to yield 152 mg of a brown semisolid. Crude **14** was purified by thin-layer chromatography (silica gel, 2000 μ, ethyl acetate/hexane/acetic acid, 25:70:5) to yield 103 mg of white crystals: mp 132 °C (dec); purity, 99% (TLC); IR (KBr, cm^{-1}) 3680−2500 (m, b, OH), 3285 (s, NH), 1720 (s, C═O); GC/MS (methyl ester) t_r = 10.9, *m*/*z* = 355 (M^+), 192, 115.

Preparation of Hapten-Activated Esters. C2-CDA, C4-CDA, or Phe-CDA (0.1−0.6 g) and *N*-hydroxysuccinimide (0.1−0.26 g) were dissolved in THF. The solution was cooled to 0 °C, and *N*,*N*′-dicyclohexylcarbodiimide (50−500 mg) was added with stirring. The reaction was continued at −20 °C for 24 h. The cold solution was filtered and methylene chloride added to the filtrate, which was washed with dH_2O and brine and dried (Na_2SO_4). Removal of solvent yielded an amber, oily crystalline residue. The esters were purified by thin-layer chromatography (20 × 20 cm, 2000 μm silica, 25% ethyl acetate/hexane). C2-CDA: IR (neat, cm^{-1}) 3320 (w, NH), 1830, 1795, 1750, 1690 (s, C═O), 1460−1370 (m, CN), 1240−1200 (s, −CO·OR); ^1H NMR ($CDCl_3$) δ 1.2 (t, 6H, $-CH_3$), 2.6 (q, 4H, alkyl $-CH_2$), 2.7 (s, 4H, imide $-CH_2$), 3.7 (s, 2H, $-NCH_2$-COOR), 4.5 (s, 2H, $ClCH_2CO{\cdot}R$), 7.2 (m, 3H, aryl CH); MS (direct probe) *m*/*z* 380 (M^+), 266, 238, 216, 188, 162. Elemental analysis (theoretical values in parentheses): C, 57.13% (56.77%); H, 5.85% (5.56%); N, 7.0% (7.36%); O, 21.08% (21.00%); Cl, 8.93% (9.30%). C4-CDA: IR (neat, cm^{-1}) 3400−3300 (w, NH), 1795, 1760, 1740, 1650 (s, C═O), 1460−1320 (m, CN), 1200 (s, −CO·OR). Elemental analysis (theoretical values in parentheses): C, 59.58% (59.04%); H, 6.43% (5.70%); N, 6.69% (6.89%); O, 18.85% (19.66%); Cl, 8.45% (8.71%). Phe-CDA IR (neat, cm^{-1}) 3290 (s, NH), 1795, 1760, 1670, 1630 (C═O), 1210 (s, CO·OR).

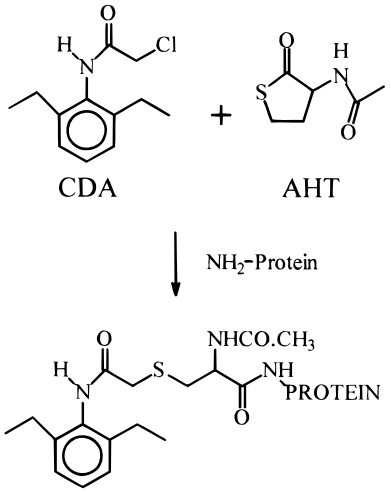

Figure 5. Direct coupling of CDA to protein carrier molecules via the coupling reagent AHT. The carrier protein was either BSA (immunogen) or OVA (plate-coating antigen).

Conjugation of Haptens to Carrier Proteins. The activated esters of C2-CDA, C4-CDA, and Phe-CDA, dissolved in THF, were stirred with carrier protein [bovine serum albumin (BSA), human serum albumin (HSA), or ovalbumin (OVA)] in phosphate-buffered saline (PBS) at 0 °C, at a 50:1 molar ratio. The reaction was continued for 48 h at 4 °C. The conjugate solutions were dialyzed against dH_2O (8−10 changes) at 4 °C and then lyophilized.

CDA was also directly coupled to carrier protein via *N*-acetyl homocysteinethiolactone (AHT; Figure 5; Feng et al., 1992). BSA or OVA (200 mg) was dissolved in dH_2O. AHT (18.4 mg, 115 mmol) was added to the protein with stirring and the solution chilled to 0 °C. CDA dissolved in dioxane was added dropwise and the reaction mixture adjusted to pH ≥10 with carbonate−bicarbonate buffer (1.0 M, pH 11). After stirring at 0 °C for an additional 15 min, the solution was transferred to a 50 °C water bath and the reaction continued for 2 h. The reaction solution was neutralized with phosphate buffer (1.0 M, pH 7.2) and dialyzed against dH_2O (eight changes).

The immunogens utilized BSA as the carrier protein, whereas the plate-coating antigens utilized OVA or HSA as the carrier protein. Hapten densities were determined by titrating free amine moieties with trinitrobenzene sulfonic acid (TNBS; Habeeb, 1965).

Antibody Production. Immunizations and blood collections were performed by Animal Care and Research Services, Graduate School, University of Massachusetts, Amherst, MA. Standard immunization protocols were followed (Coligan et al., 1995). Female white New Zealand rabbits (2−3 kg) were initially immunized with 250 μg of hapten−protein conjugate solubilized in 250 μL of PBS (pH 7.2) and emulsified 1:1 with Freund's complete adjuvant. Subsequent boosts of 250 μg of

hapten−protein conjugate in PBS, emulsified with Freund's incomplete adjuvant, were administered at 3 week intervals. Serum was collected by centrifugation and diluted 1:1 with glycerol. Thimerosal was added at 0.01% as a preservative, and the sera were stored at −20 °C.

Enzyme Immunoassays. Immunoassays were conducted on 96-well microtiter plates (Nunc). Plates were coated with hapten−protein conjugate in carbonate−bicarbonate buffer (0.05 M, pH 9.6, 50 μL/well) by incubating overnight at room temperature. After washing, plates were blocked with protein [0.25% OVA or HSA in PBS amended with 0.05% Tween-20 (PBST)]. The plates were washed again and air-dried. Dried plates were stored in zip-lock bags at 4 °C.

Optimal concentrations of antisera, plate-coating antigen, and secondary reporter antibodies were determined by checkerboard assays utilizing standard procedures as outlined in Coligan et al. (1995) and Harlow and Lane (1988). Antisera diluted in PBST were added to the microtiter plate (50 μL/well) and incubated at 37 °C for 60 min. After washing, bound antibodies were quantified using goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (anti-rabbit IgG-HRP; 50 μL/well), incubated for 30 min at 37 °C. The tetramethylbenzidine−peroxide substrate system (TMB−H_2O_2) for HRP was prepared by diluting 200 μL of TMB stock solution (25 mM in DMSO) and 17 μL of H_2O_2 (3% solution) in 12.5 mL of sodium acetate buffer (0.1 M, pH 5.5). TMB−H_2O_2 (100 μL/well) was incubated for 10−25 min at room temperature and the reaction stopped by adding 50 μL/well 4 M H_2SO_4. OD was determined at 450 nm. In competition assays, solutions of CDA, HDA, and cross-reactivity test compounds (from methanol or acetone stock solutions) were serially diluted in dH_2O to concentrations of 100, 33.3, 11.1, 3.7, 1.2, 0.4, 0.14, 0.04, 0.015, and 0.005 mg/L. Competitor solutions were added to the coated wells immediately prior to addition of primary antisera dilutions. The limit of detection was determined as the concentration that resulted in an OD value 3 standard deviations below the dH_2O control mean OD value (Brady, 1995).

Assay Optimization. Antiserum cross-reactivities to structurally related compounds were determined by competition assays over the concentration range 100 to 0.4 mg/L. The IC_{50} values (i.e., the competitor concentration required to achieve 50% inhibition of the maximal OD value) were interpolated from plots of $\%B/B_0$ versus concentration, where $\%B/B0$ is the ratio of competitor versus control OD. Percent cross reactivity ($\%R$) was quantitated as $\%R = [IC_{50}(CDA)/IC_{50}(test\ compound)] \times 100$. The effect of pH on assay performance was determined with pH-adjusted dH_2O over the pH range 4.0−9.5 in 0.5 pH increments. The standard cELSIA format was run with no analyte present (i.e., pH-adjusted dH_2O only) or with a concentration of CDA that resulted in 50% inhibition of antibody binding. To determine the effect of solvents on assay performance, the standard cELSIA format was run with standards of CDA over the concentration range 0.1−10 mg/L made up in 10, 25, or 50% methanol, ethanol, acetone, or acetonitrile in dH_2O.

Solid-Phase Extraction of Aqueous Samples. CDA and HDA were extracted from aqueous samples using Oasis HLB (3 cm^3, 60 mg) solid-phase extraction cartridges (Waters, Medford, MA). Fortified samples were prepared by fortifying tap water with stock solutions of CDA or HDA (1.0 g/L in acetone). The cartridges were conditioned with 6 mL of methanol followed by 6 mL of dH_2O. Aqueous samples (100−1000 mL) were loaded onto the columns at a flow rate of 5−10 mL/min. The cartridges were dried by maintaining the vacuum for 5 min, and CDA or HDA residues were eluted with 1.0 mL of methanol. The extracts were reduced under nitrogen to <100 μL and brought to a final volume of 1.0 mL in dH_2O for cELISA analysis or injected undiluted for analysis by GC/MS.

Chromatographic Analysis of Aqueous Sample Extracts. CDA- and HDA-fortified aqueous samples were split for analysis by GC/MS and by cELISA to determine the accuracy of the cELISAs. Samples were prepared by solid-phase extraction for chromatographic analysis. Samples in the concentration range 0.1−10 mg/L were analyzed directly by cELISA. Samples in the concentration range 0.01−10 μg/L were preconcentrated by solid-phase extraction prior to cELISA analysis.

Table 1. Percent Cross-Reactivity[a] of Antisera to Chloroacetanilide and Related Compounds

	immunogen			
compound	1	2	3	4
CDA	100	100	100	100
HDA	−[b]	−	<0.1	100
alachlor	150	≥100	<0.1	0.7
2,6-DEA	−	−	<0.1	2.0
hydroxyalachlor	−	−	<0.1	0.7
alachlor-ESA	−	−	<0.1	<0.2
acetochlor	−	−	<0.1	<0.2
butachlor	−	−	<0.1	<0.2
metolachlor	−	−	<0.1	<0.2
propachlor	−	−	<0.1	<0.2

[a] $\%R = [IC_{50}(CDA)/IC_{50}(test\ compound)] \times 100$. [b] Not tested.

RESULTS

Antibody Response to Hapten Design. Immunogens 1−4 stimulated the production of antisera that recognized the target analytes CDA and/or HDA in competition assays. Antisera against immunogens 1 and 2, however, recognized the parent compound alachlor with affinity equal to or greater than that of the intended target analyte (Table 1). Therefore, these antisera were not further characterized or utilized in ELISA development. Antisera against immunogens 3 and 4 specifically detected CDA (immunogen 3) or CDA and HDA in combination (immunogen 4) with minimal cross-reactivity to alachlor, other primary alachlor metabolites, and other chloroacetanilide herbicides (Table 1). The antisera raised against immunogens 3 and 4 were utilized in the development and optimization of functional cELISAs for the detection of CDA (cELISA I) and CDA and HDA in combination (cELISA II), respectively.

cELISA I: Antisera against Immunogen 3. Immunogen 3 was synthesized by covalent linkage of CDA to BSA carrier protein via a five-carbon spacer group attached to the aromatic ring of CDA. The corresponding plate-coating antigen utilized the same chemistry except that BSA was replaced by OVA as the carrier protein. Microtiter plates were coated with antigen at 1.0 ng/well. Antiserum to immunogen 3 was diluted 1:20000 in PBST and incubated at 37 °C for 60 min. Secondary antibody (anti-rabbit IgG−HRP) was diluted 1:20000 in PBST and incubated at 37 °C for 30 min. The plate-coating antigen concentration and dilutions of primary and secondary antibodies were determined by checkerboard assay to give an optical density signal of between 1.0 and 1.5 for maximum antibody binding (i.e., in the absence of competitors) and were used throughout all subsequent assays.

The linear range of CDA detection was 0.015−10 mg/L with an IC_{50} value of 2 mg/L (Figure 6A). Related chloroacetanilide, acetanilide, and aniline compounds did not inhibit antibody binding over the concentration range 0.4−100 mg/L (Table 1). Significantly, even HDA, which differs from CDA only in replacement of the chlorine atom with a hydroxyl moiety, was not able to bind to the CDA-specific antibodies. The correlation of fortified aqueous sample concentrations of CDA as determined by GC/MS to concentrations determined by cELISA I in the range 0.01−1 μg/L showed good agree-

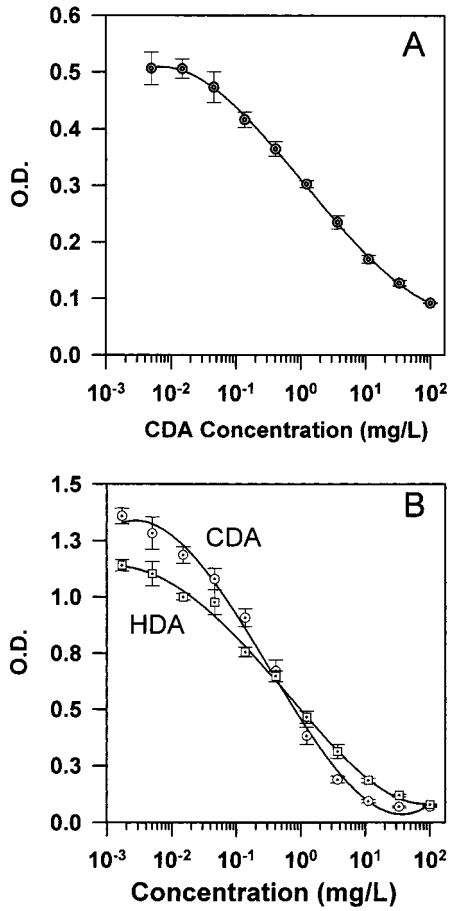

Figure 6. Competition curves for CDA in cELISA I (A) and CDA/HDA in cELISA II (B). Absorbance values (OD) are the mean of eight replicates. Error bars represent ±1 SD.

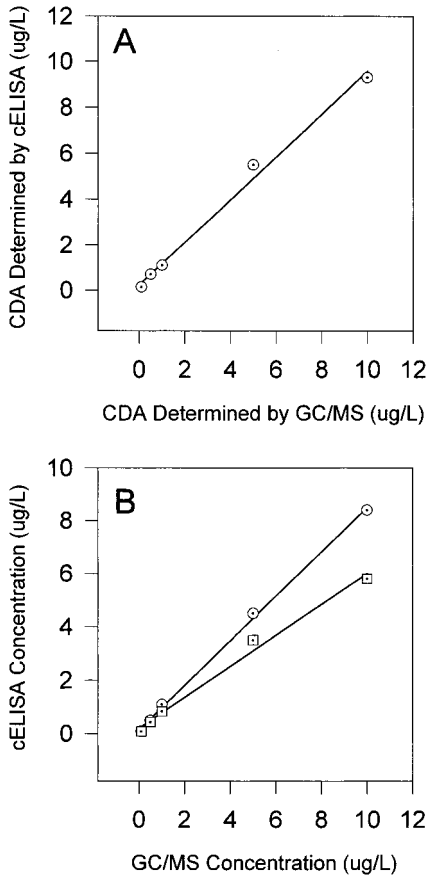

Figure 7. Correlation of cELISA with GC-MSD: (A) CDA detection by cELISA I ($y = 0.250 + 0.933x$, $r^2 = 0.992$); (B) CDA and HDA detection by cELISA II [CDA (circles), $y = 0.147 + 0.835x$, $r^2 = 0.998$; HDA (squares), $y = 0.211 + 0.579x$, $r^2 = 0.990$]. The analyte concentrations were 0.01−10 mg/L. The data are the average of three replicates with relative standard deviations <10%.

ment (Figure 7A). [Note: Sample extraction of CDA via solid-phase extraction is discussed below.]

The effect of pH on assay performance was determined with pH-adjusted dH_2O over the pH range 4.0−9.5 in 0.5 pH increments. The standard cELSIA format was run with no analyte present (pH-adjusted dH_2O only) or with 2 mg/L CDA. The inhibition of antiserum against immunogen 3 by CDA was unaffected by pH over the entire range tested (Figure 8A). To determine the effect of solvents on assay performance, the standard cELISA format was run with standards of CDA over the concentration range 0.1−10 mg/L made up in 10, 25, or 50% (v/v) methanol, ethanol, acetone, or acetonitrile in dH_2O. Methanol and ethanol concentrations up to 10% did not adversely affect the OD signal generated (Table 2). However, higher concentrations of these solvents, and as little as 10% acetone or acetonitrile, resulted in an OD signal reduction of 20−60%. Therefore, use of these solvents for extraction processes prior to analysis by cELISA I would require solvent removal and reconstitution in dH_2O or buffer.

cELISA II: Antisera to Immunogen 4. Synthesis of immunogen 4 was accomplished by thiolation of amine moieties on BSA carrier protein using *N*-acetylhomocysteine thiolactone. The thiol groups allowed spontaneous coupling of hapten to protein via the reactive chlorine atom of CDA, with resultant loss of the chlorine atom from the hapten−carrier conjugate. This linkage strategy, therefore, eliminated the only distinguishing structural feature between CDA and HDA (i.e., the chlorine atom and the hydroxyl group, respectively).

Optimal concentrations of plate-coating antigen, antiserum, and secondary antibody were determined by checkerboard assay as previously described. Microtiter plates were coated with 1.0 ng/well antigen. Antiserum to immunogen 4 was diluted 1:50000 in PBST and incubated at 37 °C for 60 min. Goat anti-rabbit IgG−HRP was diluted 1:20000 in PBST and incubated at 37 °C for 30 min. As expected, antiserum produced in response to immunogen 4 recognized both CDA and HDA with approximately equal affinity. The linear range of detection of cELISA II for CDA and HDA was 0.01−10 mg/L with an IC_{50} value of 0.2 mg/L (Figure 6B). The correlation of fortified aqueous sample concentrations of CDA and HDA as determined by GC/MS to concentrations determined by cELISA II in the range 0.01−1 μg/L showed good agreement (Figure 7B), although HDA concentration was underestimated. Cross-reactivity to alachlor, hydroxyalachlor, and 2,6-diethylaniline was minimal (0.7−2.0%), and there was no cross-reactivity to alachlor-ESA, acetochlor, butachlor, metolachlor, and propachlor (Table 1). The inhibition of antibody binding by CDA and HDA was optimal between pH 7 and 8 but was not adversely affected by pH over the entire range tested (Figure 8B). Methanol and acetone did not adversely affect assay performance, resulting in only 5−15% signal loss at concentrations up to 50% (Table 2). However, ethanol resulted in a 25−

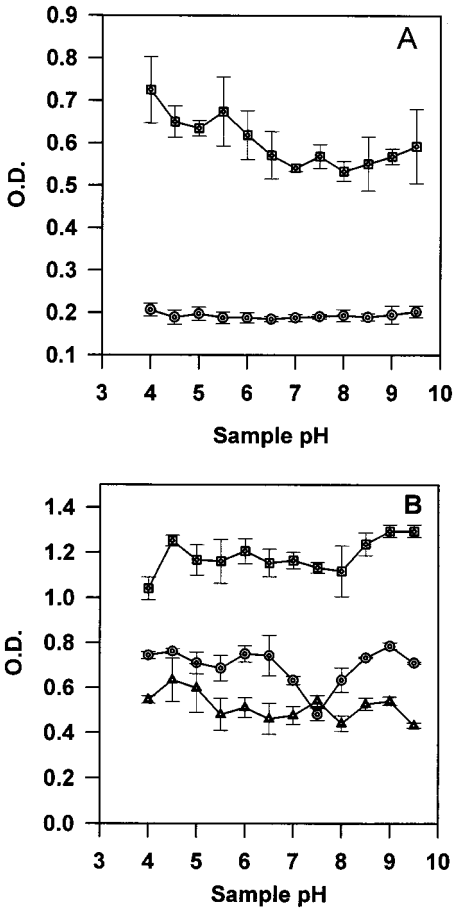

Figure 8. Effect of pH on antibody inhibition by CDA (cELISA I; A) and CDA/HDA (cELISA II; B). Samples of CDA (circles), HDA (triangles), or no competitor control (squares) in pH-adjusted water were analyzed under standard assay conditions.

Table 2. cELISA Signal Attenuation Due to the Effects of Solvents on Antibody Binding

solvent concn	% signal reduction[a]			
	methanol	ethanol	acetone	acetonitrile
cELISA I				
10%	2.6 ± 2.3	11.5 ± 5.8	19.8 ± 10.0	26.9 ± 10.8
25%	18.4 ± 2.4	37.2 ± 3.6	21.4 ± 11.8	39.8 ± 9.6
50%	20.5 ± 3.1	61.4 ± 3.3	17.2 ± 8.6	57.7 ± 9.7
cELISA II				
10%	7.8	25.2	14.1	38.2
25%	4.7	30.3	14.7	31.1
50%	5.7	63.4	12.9	37.6

[a] Percent reduction in OD compared to dH_2O control. cELISA I values, average ($n = 8$) ± SD. cELISA II values, average ($n = 2$), coefficient of variation <10%.

64% loss of signal and acetonitrile resulted in a 31−38% signal loss.

Trace Enhancement of CDA and HDA from Aqueous Samples via Solid-Phase Extraction. CDA and HDA were quantitatively recovered from aqueous samples in the range 0.01−0.5 μg/L by solid-phase extraction (Table 3). Thus, extraction of 1 L aqueous samples resulted in an analyte concentration factor of 1000. When this sample preconcentration is used, the limit of detection for CDA by cELISA I is 15 ng/L. The limit of detection for CDA and HDA by cELISA II is 10 ng/L. Therefore, the combination of solid-phase extraction and cELISA detection was suitable for the part per trillion analysis of CDA and HDA from water samples.

Table 3. Recovery of CDA and HDA from Fortified Water Samples by Solid-Phase Extraction

CDA/HDA concn (ng/L)	CDA % recovery[a]	HDA % recovery[a]
10.0	107.3 ± 16.0	88.0 ± 4.5
20.0	100.0 ± 25.8	85.1 ± 7.0
50.0	113.2 ± 1.7	88.5 ± 1.7
500.0	113.8 ± 11.0	87.0 ± 4.8

[a] Recoveries determined by GC/MS ± SD, $n = 3$.

DISCUSSION

Hapten Design. The CDA and HDA metabolites of alachlor occur in environmental samples that also contain measurable levels of alachlor. Therefore, our primary objective was to develop cELISAs that would specifically detect CDA and HDA without interference from alachlor or other potentially cross-reacting chloroacetanilide compounds, as a means of studying the environmental fate of these genotoxic compounds. We synthesized four distinct haptenic derivatives of CDA to determine the critical structural differences between CDA and alachlor that would result in antibody recognition of the target analyte alone. Because the HDA metabolite is formed via hydrolysis of the chlorine atom of CDA, we reasoned that the route of synthesis for CDA haptens would be suitable for corresponding HDA haptens with the addition of a hydrolysis step to the synthetic pathway.

The structural differences between CDA and HDA, alachlor, other alachlor metabolites, and other chloroacetanilide herbicides are (1) the degree of substitution at the amide moiety and (2) alkyl substitution at the 2- and 6-positions on the aromatic ring (Figure 1). The parent herbicides are fully substituted at the nitrogen atom (i.e., they are tertiary amides), whereas CDA, HDA, and most other metabolites are secondary amides. The single primary amine metabolite of alachlor, 2,6-diethylaniline, is a putative terminal metabolite. Alachlor and butachlor (and their metabolites) have ethyl substitutions at the 2- and 6-positions of the aromatic ring. Acetochlor and metolachlor (and their metabolites) have one ethyl and one methyl substitution at these positions. Propachlor (and its metabolites) contains a nonsubstituted aromatic ring. Therefore, the degree of substitution at the amide moiety and the type of alkyl substitution on the aromatic ring would be expected to govern any potential cross-reactivity of chloroacetanilide, acetanilide, and aniline compounds to CDA and HDA specific antibodies.

Immunogens 1 and 2 featured a two- or four-carbon linkage group, respectively, attached to the nitrogen atom of CDA. The use of the nitrogen atom as the linkage site had the advantage of straightforward synthetic procedures to produce the desired hapten. However, antisera produced in response to immunogen 1 demonstrated greater affinity for alachlor than for CDA. The length of the linkage group also influences hapten presentation for antibody recognition (Goodrow et al., 1995). Therefore, immunogen 2 was synthesized with a four-carbon spacer to replace the two-carbon spacer of immunogen 1. Antisera produced in response to immunogen 2 showed a greater affinity to CDA relative to immunogen 1 antisera. However, the antisera still demonstrated approximately equal affinities for alachlor. Utilizing the nitrogen atom of CDA for the linkage site to carrier protein produced haptens containing a tertiary amide moiety that more closely

resembled alachlor than CDA, a secondary amide. Thus, these initial efforts at synthesis of a CDA immunogen demonstrated clearly that the degree of substitution of the hapten amide moiety was crucial for selective antibody recognition.

The design of immunogens 3 and 4 preserved the secondary amide of the target analyte in the hapten−carrier conjugate. Immunogen 3 featured a five-carbon linkage group attached to carbon 4 of the aromatic ring of CDA via an ether linkage. This linkage strategy was used successfully by Schlaeppi et al. (1991) for the synthesis of a haptenic derivative of metolachlor. The attachment of a linker group directly to the aromatic ring involved a more complicated synthesis relative to immunogens 1 and 2, but the resulting hapten preserved the secondary amide moiety. The length of the linkage group also ensured that the target structure was physically distant enough from the carrier protein surface that the alkyl side chains on the aromatic ring could also contribute to antibody recognition. Antiserum produced in response to immunogen 3 specifically recognized CDA but did not cross-react with alachlor, HDA, other alachlor metabolites, or other chloroacetanilide herbicides. Significantly, this antiserum demonstrated adequate specificity to distinguish between the chlorine atom of CDA and the hydroxyl moiety of HDA, the sole difference between these two molecules. The lack of cross-reactivity to alachlor-ESA is also significant because this compound was found to be a major cross-reactive interference with commercial alachlor immunoassays that resulted in a number of "false-positive" reports for alachlor (Baker et al., 1993).

To further demonstrate that the degree of substitution of the amide moiety is a critical parameter of hapten design for acetanilide-type compounds, we utilized the chlorine atom of CDA as a third attachment point for conjugation to protein molecules in immunogen 4. The use of the chlorine atom of CDA as a linkage site avoided the synthesis of a unique hapten altogether by allowing direct coupling of CDA to BSA through the coupling reagent AHT. Feng et al. (1992) successfully utilized this approach to create hapten−carrier conjugates for a variety of chloroacetanilide herbicides. Although immunogen 4 preserved the secondary amide state in the hapten, this linkage strategy eliminated the only distinguishing structural feature between CDA and HDA (i.e., the chlorine atom and the hydroxyl group, respectively). Therefore, antisera produced in response to immunogen 4 recognized both CDA and HDA with equal affinity but distinguished these compounds from alachlor, other alachlor metabolites, and other chloroacetanilides. The minimal cross-reactivity of immunogen 4 antiserum to alachlor, hydroxyalachlor, and 2,6-diethylaniline indicates that in addition to the substitution state of the amide, the ethyl substitution at the 2- and 6-positions of the aromatic ring also plays a role in antibody recognition. This secondary role of ring substitution would presumably minimize cross-reactivity by metabolites of other chloroacetanilide herbicides, although these compounds were not available for testing by us.

In conclusion, these results clearly indicate that for the production of antisera specific for acetanilides, the degree of substitution of the amide moiety is of primary importance and must be preserved in the hapten design. Alkyl substitution in the aromatic portion of the molecule is of secondary importance, but does play a role in antibody recognition.

The CDA and CDA/HDA cELISAs. cELISA I was based on antiserum to immunogen 3, which specifically detected CDA in the range of 0.015−10 mg/L, with an IC_{50} of 2 mg/L. Different protein carriers were used for raising antisera and for the plate-coating antigen. This ensured that only CDA specific antibodies would react in the assay and that antibodies that recognized the BSA carrier protein would not cause interference and compromise sensitivity. However, antiserum pools were not purified prior to use [e.g., via affinity chromatography (Szekacs and Hammock, 1995)], so this cELISA was not at the limit of its sensitivity.

cELISA II was based on antiserum to immunogen 4, which specifically detected CDA and HDA in the range 0.01−10 mg/L, with IC_{50} values of 0.5 mg/L for CDA and 0.3 mg/L for HDA. This assay also utilized different carrier proteins for the immunizing and plate-coating conjugates. Identical competition curves for CDA and HDA would be expected on the basis of the hapten design. The slight differences in the shapes of the competition curves and for the IC_{50} values are likely due to differences in the water solubilities, antibody−analyte hydrogen-bonding potential, steric differences between CDA and HDA (i.e., the chlorine of CDA versus the hydroxyl group of HDA), and the response of different portions of the polyclonal antibody pool to each compound.

Solid-phase extraction procedures were developed for extraction of CDA and HDA from aqueous samples. It was possible to effect a 1000-fold concentration of both compounds. Thus, the effective detection limit for CDA and HDA in aqueous samples was lowered to 15 parts per trillion (pg/mL) by SPE/cELISA analysis.

Ground water or surface water samples potentially exhibit a range of pH values, so it was important to assess the influence of pH on the assay. The pK_a values of CDA and HDA are unknown, but for the parent aniline the $pK_a \geq 27$ (Harris and Hayes, 1982). Therefore, the amide moieties of CDA and HDA are unlikely to be ionized, so any potential influence of pH on the assay would be on antibody binding rather than on the ionization state of the target analytes. cELISA I and cELISA II produced stable results in solutions that had pH values adjusted between 4 and 9, due to the buffering capacity of the antiserum solution buffer.

Polar solvents are utilized for the extraction of CDA and HDA from soil and other environmental samples, as well as in the solid phase extraction procedures used for aqueous samples. cELSIA I is adversely affected by the solvents acetone and acetonitrile but is stable to low concentrations (i.e., ≤10%) of methanol and ethanol. cELISA II was stable to sample concentrations of ≤50% methanol or acetone but was adversely affected by the solvents ethanol and acetonitrile.

The synthesis of various haptenic derivatives has demonstrated the key structural elements governing antibody recognition and selectivity toward chloroacetanilide herbicide metabolites. The availability of CDA and HDA specific antibodies provides a basis for both cELISA detection and affinity-type preconcentration for instrumental analysis. This will enable a more comprehensive analytical assessment of these genotoxic metabolites of alachlor and of the overall environmental impact of this heavily utilized herbicide.

ABBREVIATIONS USED

AHT, *N*-acetyl homocysteinethiolactone; BSA, bovine serum albumin; CDA, 2-chloro-2′,6′-diethylacetanilide;

cELISA, competitive enzyme-linked immunosorbent assay; DCC, *N*,*N*′-dicyclohexylcarbodiimide; dH_2O, distilled water; GC/MS, gas chromatography/mass spectrometry; HDA, 2-hydroxy-2′,6′-diethylacetanilide; 2,6-DEA, 2,6-diethylaniline; ^1H NMR, proton magnetic resonance spectroscopy; IC_{50}, 50% inhibition concentration; IR, infrared spectroscopy; NHS, *N*-hydroxysuccinimide; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, PBS-−0.05% Tween 20; THF, tetrahydrofuran; TMB, tetramethylbenzidine; TNBS, trinitrobenzene sulfonic acid.

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