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Determination of Metolachlor by Competitive Enzyme Immunoassay Using a Specific Monoclonal Antibody

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A carboxylic derivative of metolachlor, *N*-(chloroacetyl)-*N*-(1-methyl-2-methoxyethyl)-2-methyl-4-(4′-carboxybutoxy)-6-ethylaniline, was synthesized and covalently linked to a carrier protein to prepare monoclonal antibodies (MAbs). One antibody with high binding affinity for metolachlor was used to develop two competitive ELISA procedures, a direct one with enzyme-labeled antigen and an indirect one with an enzyme-labeled second antibody. The detection of metolachlor in aqueous media ranged from 0.1 to 10 ppb. The MAb was specific for metolachlor. No cross-reactivity was observed with alachlor, furalaxyl, metalaxyl, and most of the metabolites of metolachlor and metalaxyl. Analysis of fortified soil samples by direct and indirect ELISA showed average recoveries of 98% and 89%, respectively. Interferences due to soil matrix effects were minimal.

INTRODUCTION

Metolachlor is the active ingredient of Dual, a selective herbicide widely used in corn, cotton, potato, and sugar cane plantations. Its determination in soil and water samples is currently done by GLC or HPLC and requires time-consuming sample preparation to reach the generally accepted limits of detection of 0.1 ppb in water and 20 ppb in soil (Van Rensburg, 1985; Brooks et al., 1989; Huang, 1989; Ramsteiner, 1989). These cleanup steps could be avoided by using immunoassays as an alternative approach to residue analysis. Such immunochemical determinations, based on competitive binding of residues to an antibody, were recently developed for the determination of several herbicides and other pesticides [for reviews, see Hammock and Mumma (1980); Vanderlaan et al. (1988), Van Emon et al. (1989); Jung et al. (1989), and Hall et al. (1990)]. Two immunoassays for the detection of chloroacetanilide herbicides and related compounds were reported recently, one for the detection of alachlor at the parts per billion level in water samples (Feng et al., 1990) and one for the determination of metalaxyl in foods (Newsome, 1985). To prepare metolachlor MAbs, we synthesized a hapten designed for the production of highly specific antibodies. The results obtained with fortified soil samples indicate that our MAb-based ELISA is capable of detecting metolachlor down to the parts per billion level. By use of the hybridoma technology, an unlimited supply of reagents is guaranteed.

EXPERIMENTAL PROCEDURES

Materials. Metolachlor (2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide) and the analogues were synthesized in the laboratories of Ciba-Geigy Ltd. (Basle, Switzerland). Alkaline phosphatase from calf intestine (EIA grade) was purchased from Calbiochem. All the other reagents and cell culture media were obtained as described previously (Schlaeppi et al., 1989).

Hapten Synthesis. The synthesis of *N*-(chloroacetyl)-*N*-(1-methyl-2-methoxyethyl)-2-methyl-4-(4′-carboxybutoxy)-6-ethylaniline was carried out in six steps as shown in Figure 1.

3-Ethyl-4-nitroso-5-methylphenol (1). Concentrated HCl (600 mL) was added at room temperature to a stirred solution of 3-ethyl-5-methylphenol (102 g, 749 mmol) in 600 mL of ethanol. The reaction mixture was cooled to 0 °C and $NaNO_2$ (77.5 g, 1123 mmol in 78 mL of deionized water) was added. After 2 h of additional stirring at 5 °C, the solution was poured into ice–water. The resulting precipitate was filtered, washed with ice–water, and recrystallized from methanol, giving 88.7 g of 1 (72%), mp 138 °C (decomposition).

3-Ethyl-4-amino-5-methylphenol (2). Compound 1 (88.7 g, 537.6 mmol in 1 L of tetrahydrofuran) was hydrogenated at normal pressure in the presence of 10 g of palladium catalyst (5% palladium on charcoal). *Warning*: Due to fire hazard, caution must be taken in handling the palladium catalyst. The catalyst must be immersed in a CO_2 atmosphere to displace the oxygen before the tetrahydrofuran is added. A total of 77% of the calculated hydrogen was absorbed. The catalyst was removed by filtration. The solution was evaporated to dryness, and the residue was recrystallized from methanol, giving 51.2 g of 2 (63%), mp 167–170 °C. The elemental analysis of 2 gave the following results: $C_9H_{13}NO$ (151.21), percent calculated vs percent found, C, 71.49 vs 71.5; H, 8.67 vs 8.7; N, 9.26 vs 9.3.

2-Methyl-4-[4′-(ethoxycarbonyl)butoxy]-6-ethylaniline (3). Compound 2 (51.2 g, 339 mmol, dissolved in 500 mL of DMSO at room temperature) was cooled to 15 °C before KOH 85% (33.5 g, 508 mmol) was added. The temperature was maintained below 20 °C. Ethyl 5-bromovalerate (82.7 mL, 508 mmol) was added to the stirred reaction mixture over a period of 70 min at 25 °C. The solution was poured into 4 M HCl (130 mL in crushed ice) under stirring. The aqueous solution was washed with diethyl ether, then basified with 2 M NaOH, and finally reextracted with diethyl ether. The ethereal solution was washed with water and brine, dried over Na_2SO_4, and evaporated to dryness, giving 67.5 g of a viscous oil which was distilled, bp 151–152 °C/0.01 Torr, to yield 31.7 g of 3 (33.7%).

***N*-(1-Methyl-2-methoxyethyl)-2-methyl-4-[4′-(ethoxycarbonyl)butoxy]-6-ethylaniline (4).** Methoxy-2-propanone 72% (20 g, 163 mmol in methanol) was added dropwise at room temperature to compound 3 (30.4 g, 108.8 mmol, in 300 mL of methanol containing 0.3 g of H_2SO_4). The reaction mixture was hydrogenated at 500 kPa over 8 h at 40 °C in the presence of 1.5 g of platinum catalyst (5% platinum on charcoal). A total of 107% of the calculated hydrogen was absorbed. The catalyst was filtered, and the solution was evaporated to dryness. The residue was dissolved with diethyl ether, and the ethereal solution was washed with a $NaHCO_3$ solution, ice–water, and brine, dried over Na_2SO_4, and evaporated to dryness, giving 29 g (75.9%) of a light brown oil.

***N*-(1-Methyl-2-methoxyethyl)-2-methyl-4-(4′-carboxybutoxy)-6-ethylaniline (5).** Compound 4 (19 g, 54 mmol) was added to 2 M KOH (150 mL) and stirred at room temperature over 20 h. The reaction mixture was neutralized at pH 7 with 6 M HCl and reextracted twice with diethyl ether. The ethereal solution was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The yield of 5 was 12.9 g (74%); n^{23}_D 1.517. The elemental analysis of 5 gave the following results: $C_{18}H_{29}$-

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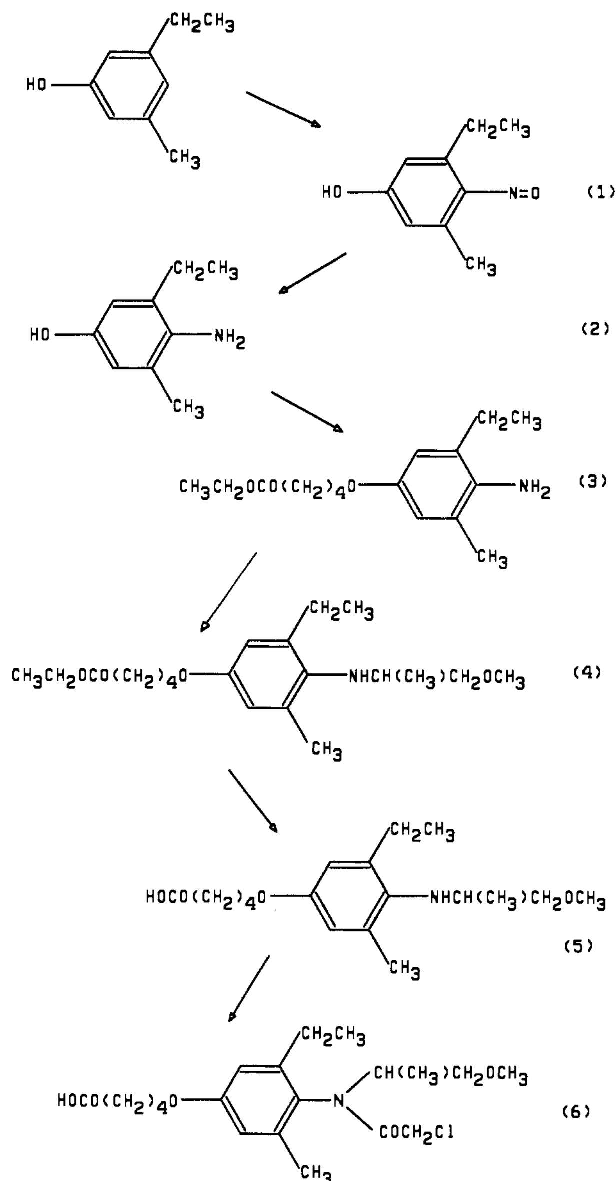


Figure 1. Synthesis of *N*-(chloroacetyl)-*N*-(1-methyl-2-methoxyethyl)-2-methyl-4-(4′-carboxybutoxy)-6-ethylaniline.

NO_4 (323.4), percent calculated vs percent found, C, 66.85 vs 66.6; H, 9.04 vs 9.3; N, 4.33 vs 4.2.

***N*-(Chloroacetyl)-*N*-(1-methyl-2-methoxyethyl)-2-methyl-4-(4′-carboxybutoxy)-6-ethylaniline (6).** NaOH (1 M, 30.6 mL) was added at room temperature to a stirred solution of **5** in dichloromethane (400 mL). Chloroacetyl chloride (2.7 mL, 33.7 mmol) was added slowly at 20–25 °C. The reaction mixture was stirred for an additional 2 h before 10.2 mL of 1 M NaOH and 0.9 mL of chloroacetyl chloride were again added. The solution was stirred for 12 h at room temperature, basified with 1 M NaOH (32 mL), and washed with diethyl ether. The aqueous solution was acidified to pH 3 with 1 M HCl (32 mL) and the reaction product extracted into diethyl ether. The ethereal phase was washed with ice–water and brine and finally dried over Na_2SO_4 and evaporated to dryness. The yield of compound **6** was 7.2 g (59%), $n^{23}{}_D$ 1.515. The structure of compound **6** was confirmed by mass spectrometry on a Finnigan 4500 instrument with a direct-exposure probe (DEP). The electron impact spectrum showed the molecular ion (399 amu, 2% relative intensity), the loss of formic acid (354 amu, 17%), the loss of $CH_2CHCOOH$ (327 amu, 11%) and the loss of COCHCl from fragment 354 (278 amu, 12.5%). The base peak at 73 amu (100%) corresponded to the structure $CH_3CHCH_2OCH_3$. Further fragments were detected at 256 (5%), 176 (5%), 162 (8%), 148 (18%), 97 (14%), 83 (24%), 69 (32%), and 60 amu (68%). The positive ionization spectrum with methane as a reagent gas showed the protonated molecular ion (400 amu, 100%), the loss of HCl (364 amu, 35%), and the loss of methanol (368 amu, 20%).

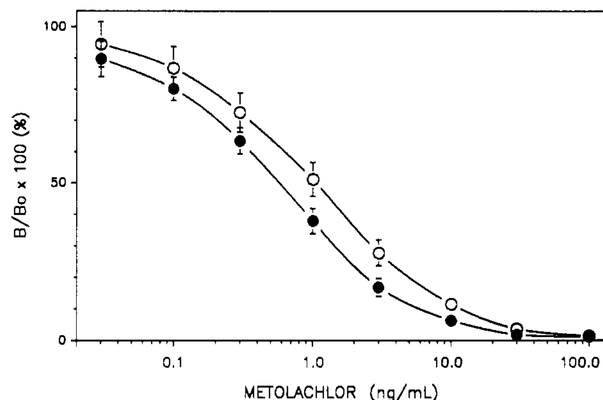


Figure 2. Indirect ELISA with BSA–hapten-coated plates and enzyme-labeled second antibody (●); direct ELISA with antibody-coated plates and enzyme–hapten conjugate (○). ($B/B_0 \times 100\%$) represents the percentage of MAb bound to the plate. Vertical bars represent standard deviations between assays ($n = 10$).

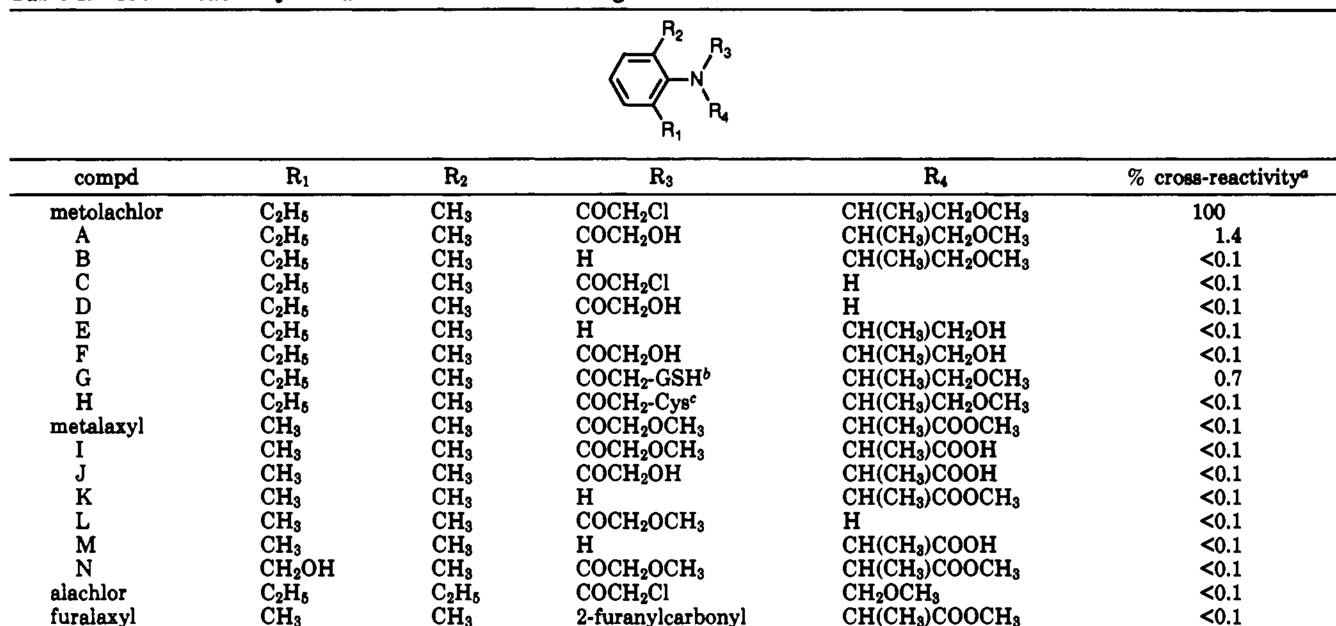
Preparation of the Hapten–Protein Conjugates. The hapten was covalently linked to bovine serum ablumin (BSA) or keyhole limpet hemocyanin (KLH) by the active ester method. Compound **6** (8 mg dissolved in 200 μL of DMF) was reacted with a 4 M excess of *N*-hydroxysuccinimide (9.1 mg/200 μL of (DMF) and *N,N*′-dicyclohexylcarbodiimide (16 mg/200 μL of DMF). The reaction mixture was stirred for 1 h at 22 °C and then for 18 h at 4 °C. The precipitate was removed by centrifugation, and the clear supernatant containing the active ester was added to BSA or KLH (24 mg) dissolved in 5.4 mL of phosphate-buffered saline (PBS) (0.01 M sodium phosphate and 0.145 M NaCl, pH 7.0). After a 4-h incubation at 4 °C, the white precipitate was removed by centrifugation and the supernatant containing the protein conjugate was extensively dialyzed in PBS containing 3 mM NaN_3 before it was used for immunization. The protein concentration was determined according to the Lowry assay (Lowry et al., 1951). The extent of coupling of the hapten to BSA was determined by UV absorbance at 280 nm, which corresponded approximately to the peaks of both BSA and compound **6** (A_{max} = 274 nm). By assuming additive absorbance values, we determined that the molar ratio of hapten to BSA was 23/1.

Preparation of the Antigen–Enzyme Conjugate. The active ester of compound **6** (1.2 μL, 16 μg; see above) was added to 40 μL of alkaline phosphatase (153 μg/230 units). After a 4-h incubation at 4 °C, the mixture was extensively dialyzed against PBS containing 3 mM NaN_3. The enzymatic activity of the conjugate was determined by using *p*-nitrophenyl phosphate as a substrate. The activity of the enzyme remained unchanged after the hapten coupling.

Immunization and Fusion Protocol. Following previous practice (Schlaeppi et al., 1989), groups of five BALB/c female mice (4–6 weeks old) were given a series of three injections with the KLH–hapten conjugate (50 μg/injection) mixed with Freund's adjuvant. Serum titers were measured by ELISA 1 week after the last injection using the BSA–hapten conjugate. After a rest period of 2 months, the mice were boosted intraperitoneally with 370 μg of the KLH–hapten conjugate in PBS (200 μL); 3–4 days later, the mice were sacrificed and the spleen cells were fused with the murine myeloma cell line Sp 2/0.Ag14 (Shulman et al., 1978) by using PEG 4000, according to a modification of the original Köhler and Milstein method (Galfre et al., 1977). After 2–4 weeks, wells containing growing hybridomas in HAT medium were tested for specific MAbs by ELISA, only the IgG isotype was taken into consideration, by using a alkaline phosphatase labeled goat antibody specific to mouse IgG. Positive hybridomas were cloned by limiting dilution (Goding, 1980), and MAbs were purified from mouse ascitic fluid (Schlaeppi et al., 1989).

Competitive ELISA Using Enzyme-Labeled Second Antibody (Indirect ELISA). The two-step competitive ELISA was done as previously described (Schlaeppi et al., 1989). The 96-well microtiter plates were coated with the BSA–hapten

Table I. Cross-Reactivity of Various Metolachlor Analogues with MAb 4082-25-4



compd	R_1	R_2	R_3	R_4	% cross-reactivity[a]
metolachlor	C_2H_5	CH_3	$COCH_2Cl$	$CH(CH_3)CH_2OCH_3$	100
A	C_2H_5	CH_3	$COCH_2OH$	$CH(CH_3)CH_2OCH_3$	1.4
B	C_2H_5	CH_3	H	$CH(CH_3)CH_2OCH_3$	<0.1
C	C_2H_5	CH_3	$COCH_2Cl$	H	<0.1
D	C_2H_5	CH_3	$COCH_2OH$	H	<0.1
E	C_2H_5	CH_3	H	$CH(CH_3)CH_2OH$	<0.1
F	C_2H_5	CH_3	$COCH_2OH$	$CH(CH_3)CH_2OH$	<0.1
G	C_2H_5	CH_3	$COCH_2$-GSH[b]	$CH(CH_3)CH_2OCH_3$	0.7
H	C_2H_5	CH_3	$COCH_2$-Cys[c]	$CH(CH_3)CH_2OCH_3$	<0.1
metalaxyl	CH_3	CH_3	$COCH_2OCH_3$	$CH(CH_3)COOCH_3$	<0.1
I	CH_3	CH_3	$COCH_2OCH_3$	$CH(CH_3)COOH$	<0.1
J	CH_3	CH_3	$COCH_2OH$	$CH(CH_3)COOH$	<0.1
K	CH_3	CH_3	H	$CH(CH_3)COOCH_3$	<0.1
L	CH_3	CH_3	$COCH_2OCH_3$	H	<0.1
M	CH_3	CH_3	H	$CH(CH_3)COOH$	<0.1
N	CH_2OH	CH_3	$COCH_2OCH_3$	$CH(CH_3)COOCH_3$	<0.1
alachlor	C_2H_5	C_2H_5	$COCH_2Cl$	CH_2OCH_3	<0.1
furalaxyl	CH_3	CH_3	2-furanylcarbonyl	$CH(CH_3)COOCH_3$	<0.1

[a] Cross-reactivity is defined as (metolachlor concentration for 50% inhibition)/(metolachlor analogue concentration for 50% inhibition) × 100. [b] GSH, *S*-glutathione. [c] Cys, *S*-cysteine.

Table II. Recovery of Metolachlor from Various Fortified Soils Determined by ELISA Using MAb 4082-25-4

	soil composition						metolachlor recovered			
							indirect ELISA		direct ELISA	
soil	% humus	% sand	% silt	% clay	pH	metolachlor added, ppb	ppb[a] (%)[b]	SD (CV)[c]	ppb (%)	SD (CV)
Vetroz (Switzerland)	9.3	18.1	60.4	21.5	7.3	20	21.2 (103)	3.7 (17.3)	25.4 (121)	4.3 (16.8)
						50	44.7 (88)	5.2 (11.6)	46.5 (91)	3.6 (7.8)
Collombey (Switzerland)	1.4	83.9	13.6	2.5	7.4	20	12.5 (62)	2.7 (21.4)	18.4 (87)	4.1 (22.1)
						50	36.9 (74)	1.4 (3.8)	46.4 (91)	4.6 (9.8)
Les Evouettes (Switzerland)	2.6	25.7	64.0	10.3	6.2	20	23.3 (112)	4.6 (19.7)	25.9 (120)	2.7 (10.4)
						50	58.6 (115)	6.8 (11.6)	50.2 (97)	4.9 (9.7)
Speyer (Germany)	1.0	93.0	3.1	2.9	7.4	20	12.2 (60)	2.2 (17.8)	20.6 (97)	3.9 (18.7)
						50	49.2 (98)	6.1 (12.4)	43.0 (84)	7.1 (16.6)

[a] Calculated according to standards made of metolachlor in PBS-Tween. [b] The percentage of metolachlor recovered is defined as [(ppb measured after addition − ppb before addition)/ppb added] × 100. [c] SD, standard deviation; CV, coefficient of variation (average of five determinations).

conjugate (50 ng/100 μL of 50 mM sodium carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plates were then washed with PBS supplemented with 0.1% (v/v) Tween 20 and blocked with PBS supplemented with 1% BSA (w/v). In separate test tubes, 50 μL of purified MAb (0.3 μg/mL) was incubated either with 950 μL of standard solutions containing increasing amounts of metolachlor or with soil extracts (both in 0.1% PBS–Tween). After a 1-h incubation at 22 °C, 200 μL of the antibody–antigen mixture was transferred to each well and incubated for an additional hour. The wells were then washed, and 100 μL of goat anti-mouse antibody conjugated to alkaline phosphatase (diluted 1:1500) was added to the wells and incubated for 1.5 h. After washing, 150 μL of the substrate *p*-nitrophenyl phosphate (1 mg/mL) in diethanolamine buffer (1 M, pH 9.8, supplemented with 0.5 mM $MgCl_2 \cdot 6H_2O$) was added to each well. After a 2-h incubation at 22 °C, the change of color, which was proportional to the amount of antibody reacting with the antigen bound to the solid phase, was monitored at 405 nm. The dilutions of the MAbs were chosen to give absorbance values, without inhibitor (B_0), between 0.3 and 0.5. All samples were done in triplicate.

Typical inhibition curves (as shown in Figure 2) were obtained by plotting $B/B_0 \times 100$ (percent bound) vs the concentration of inhibitor present (B_0 represented the absorbance measured without metolachlor inhibitor added to the antibody and B the absorbance measured with various concentrations of metolachlor inhibitor). The concentration of metolachlor or analogues, reducing the ELISA signal to 50% of the control (I_{50}) was calculated by using an adaptation of the curve fitting program ENZFITTER (R. J. Leatherbarrow, Elsevier-Biosoft) based on a four-parameter logistic curve (Raab, 1983).

Competitive ELISA Using Antigen–Enzyme Conjugate (Direct ELISA). Microtiter plates were coated with MAb 4082-25-4 (75 ng/100 μL of sodium carbonate buffer). After washing and blocking, 150 μL of either standard solutions with increasing concentrations of metolachlor or soil extracts were added to the wells and incubated for 30 min at 22 °C. Then, 50 μL of the hapten–alkaline phosphatase conjugate (2 μg/mL) was added, and the incubation was continued for an additional 30 min. After washing, 150 μL of substrate was added to each well, and the absorbance at 405 nm was monitored after 2 h (see above).

Soil Samples Analysis. Standard soil samples from various locations were fortified with standard solutions of metolachlor prepared in ethanol. The samples (10 g) were extracted by reflux for 2 h with 100 mL of methanol/water (80/20 v/v). The mixtures were filtered and evaporated. The residual soil extracts were dissolved in 50 mL of PBS–Tween 0.1% prior to the ELISA determination (see above).

RESULTS AND DISCUSSION

Preparation of Monoclonal Antibodies. As shown by previous studies, critical factors for optimal hapten design resided first in the position of the spacer arm to maximize exposure of unique portions of the molecule to antibody recognition and second in the nature of the spacer arm, usually a nonpolar bridging group two to five atoms long, ended by a functional group for covalent attachment to the carrier protein (Jung et al., 1989; Goodrow et al., 1990). Knowing these critical factors, we synthesized a metolachlor derivative with a short spacer and a carboxyl group attached in the para position to the amino group (Figure 1). In this configuration, it was supposed that the

chloroacetyl and the methylmethoxyethyl moieties could be free to interact with antibodies, allowing a maximum specificity. Indeed, it was shown previously, that antibodies prepared by using methalaxyl acid as a hapten cross-reacted with both metalaxyl and metolachlor (Newsome, 1985). However, Feng et al. (1990) reported recently the preparation of specific alachlor antibodies by coupling the chloroacetamide moiety to proteins.

Spleen cells of mice immunized with the KLH-metolachlor conjugate were fused with myeloma cells. The fusion efficiency averaged 90%. Of several fusions, we obtained seven metolachlor MAbs. One of them, MAb 4082-25-4, and IgG_1, showed a very high affinity for metolachlor by competitive ELISA. This MAb was used to develop two competitive immunoassays, one based on BSA-hapten-coated microtiter plates and enzyme-labeled goat anti-mouse second antibody (indirect ELISA) and the other using a hapten-enzyme conjugate and antibody-coated plates (direct ELISA). As shown in Figure 2, both assays could detect metolachlor at the parts per billion level. The direct ELISA was slightly less sensitive than the indirect one but was much quicker; the I_{50} values were 1.0 and 0.6 ng/mL, respectively. The minimum detectable amounts of metolachlor, defined as being the concentration required to produce a decrease in the percent of MAb bound equal to 2 times the standard deviation of the blank response, were 0.05 and 0.1 ppb by indirect and direct ELISA, respectively. The coefficient of variation between replicates at 1 ppb was 11% (20 determinations).

Cross-Reactivity of MAb 4082-25-4. The cross-reactivity pattern of MAb 4082-25-4 was examined by competitive indirect ELISA. As shown in Table I, only a weak cross-reaction was observed with the structurally closely related hydroxy metabolite of metolachlor (A) and with the glutathione metabolites (G) (1.4% and 0.7%, respectively). The remaining metabolites showed no cross-reactivity. The MAb did not bind other chloroacetanilide herbicides and related compounds such as alachlor, furalaxyl, metalaxyl, and most of the metabolites of the latter.

Soil Fortification Study. A recovery study was performed with four standard soils of known composition. The soil samples were fortified with 20 and 50 ppb of metolachlor and analyzed by both direct and indirect ELISA. As shown in Table II, all soil types gave acceptable recoveries (98% and 89% mean recoveries by direct and indirect ELISA, respectively). The overall recovery showed variations ranging from 62% to 121%. In these experiments, the interassay coefficients of variation obtained by direct and indirect ELISA were 14.5% and 14%, respectively (40 assays). The blank soil extracts (without metolachlor) showed no significant effect on the baseline response of the indirect ELISA (>90% of buffer control), whereas by direct ELISA the baseline response was slightly influenced by the extracts (>75% of buffer control), suggesting that some interferences occurred, very likely as a result of the concomitant incubation of the enzyme conjugate with the soil extracts.

In conclusion, our results indicate that the MAb 4082-25-4 based enzyme imunoassays are sensitive and selective. They constitute a valuable method for the detection of metolachlor in soil samples down to the parts per billion level, fulfilling the sensitivity requirements for environmental monitoring.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; I_{50}, concentration of metolachlor or analogues reducing the ELISA signal to 50% that of the control; KLH, keyhole limpet hemocyanin; MAb, monoclonal antibody; PBS, phosphate-buffered saline.

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