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Robust and Sensitive Monoclonal Enzyme-Linked Immunosorbent Assay for the Herbicide Molinate

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This paper reports on the generation of monoclonal antibodies and the development of a new enzyme-linked immunosorbent assay (ELISA) for the detection of molinate (*S*-ethyl hexahydroazepine-1-carbothioate). Hybridoma cells were generated using spleen and lymph node cells from a mouse immunized with *S*-2-carboxyethyl hexahydroazepine-1-carbothioate conjugated to keyhole limpet hemocyanin. After screening with a competitive ELISA, two monoclonal antibodies, mAbs 16C11 and 14D7, with IC_{50} values of 82 ± 2 and 173 ± 8 ng/mL, respectively, were selected. MAb 16C11 can detect molinate concentrations of 1 ng/mL with no cross-reactivity to any other thiocarbamate pesticides; however, it was susceptible to the presence of organic solvents and to variation in buffer ionic strength. MAb 14D7 tolerated concentrations up to 5% of propylene glycol and 12.5% of acetonitrile in the assay buffer. The sensitivity of mAb 14D7 was further improved by decreasing the amount of coating antigen in the ELISA; the final inhibition assay showed an IC_{50} of 69.2 ± 1.4 ng/mL. In summary, mAb14D7 provided a more sensitive and robust assay, as compared with previous polyclonal antibody-based assays, with the additional advantage of being based upon a consistent and unlimited source of a defined reagent.

KEYWORDS: Molinate; thiocarbamate herbicide; monoclonal antibody; ELISA

INTRODUCTION

Over the past decade, enzyme immunoassay has become an important analytical technique, particularly in the biological research and clinical medicine areas (*1*). Immunochemical approaches to environmental analyses have also experienced considerable progress, and several pesticide immunoassays have been developed over the past 10 years (*2*). The availability of an increasing number of antibodies with high affinity and selectivity, as well as improved robustness toward matrix effects, for various xenobiotics has greatly contributed to promoting the immunoassay as a powerful analytical tool. Recently, new applications, especially in the field of immunochromatography and immunosensors, have increased the demand for reliable and standardized antibodies (*3*, *4*).

Advantages and limitations of immunoassays have been discussed on numerous occasions (*5*, *6*), and identifying the limitations is an important step in the process of improving the potential of the method. The major limitations are probably in the production of suitable antibodies for new analytes and the effort necessary for standardization of immunochemical methods. The production of monoclonal antibodies by means of hybridoma technology overcomes some of the immunoassay limitations by providing an unlimited source of antibodies of the same isotype with constant properties (*7*).

In this paper we report on the production of a monoclonal antibody (mAb) against the herbicide molinate (Ordram, *S*-ethyl hexahydroazepine-1-carbothioate). Molinate is a selective, preemergence thiocarbamate herbicide used primarily in rice production (*8*). Dissipation of molinate from flooded rice fields by the drainage of rice paddies into adjacent rivers or by volatilization of molinate from water into the atmosphere (*8*) may have a toxic effect on the local fish population (*9*). In addition, contamination of drainage and rivers holds the potential for unacceptable levels of molinate in drinking water. Therefore, there is a constant need to monitor molinate content in rice field drainage canals. Since Gee et al. reported in 1988 a molinate immunoassay based on polyclonal antibodies (*10*), there have been no other studies reported in this regard. Moreover, there are no known papers on the development of monoclonal antibodies for molinate. We proposed to raise monoclonals antibodies to develop a new enzyme-linked immunosorbent assay (ELISA) that improves the assay performance achieved using polyclonal sera (*10*).

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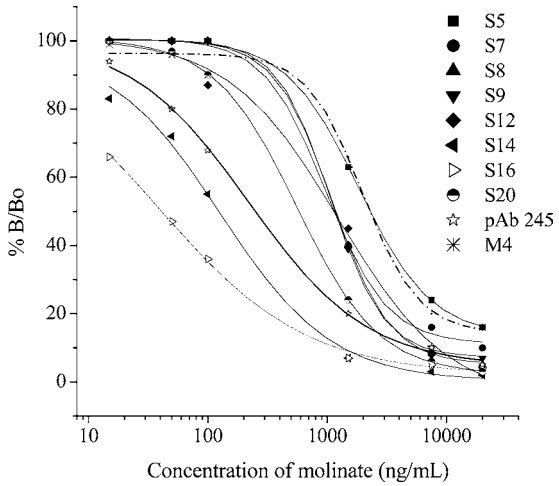


Figure 1. Inhibition curves with molinate and different hybridoma supernatants. Polystyrene 96-well microtiter plates were coated with 100 μL of 2 μg/mL **7b**–CONA. Appropriate dilutions of each supernatant (S5, S7–9, S12, S14, S16, S20), pAb 245, and mouse serum M4 were incubated with several concentrations of molinate, and the assay was done as described under Materials and Methods.

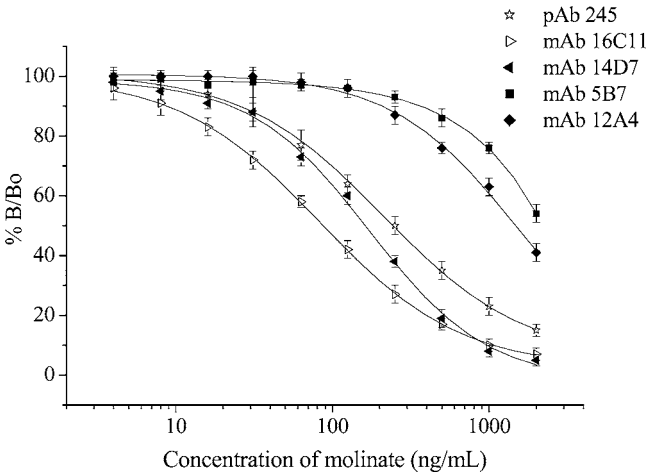


Figure 2. Inhibition curves with molinate and different anti-molinate antibodies. Polystyrene 96-well microtiter plates were coated with 100 μL of 2 μg/mL **7b**–CONA. Appropriate dilutions of each antibody (pAb 245, 1/5000; mAb 16C11, 1/200; mAb 14D7, 1/400; mAbs 12A4 and 5B7, 1/3000) were incubated with several concentrations of molinate. Each point represents the mean ± SD of at least eight independent experiments with three replicates.

MATERIALS AND METHODS

Materials. Molinate and the thiocarbamate compound standards used in the inhibition assays were gifts from Stauffer Chemical Co. Thiobencarb was a gift from Chevron Chemical Co. The molinate derivatives for immunoconjugate synthesis, *S*-2-carboxyethyl hexahydrozepine-1-carbothioate (**3a**) and *S*-2-(*p*-aminophenyl) ethyl hexahydroazepine-1-carbothioate (**7b**) (**Table 2**), and the rabbit polyclonal sera anti-molinate (pAb 245) were obtained previously in Dr. Hammock's laboratory (*10*).

Hapten—Protein Conjugates. Immunogen **3a**−keyhole limpet hemocyanin (KLH) and coating antigen **7b**−conalbumin (CONA) were synthesized by Drs. Miyamoto and Goodrow (*10*). The hapten **3a** conjugated to ovalbumin (OVA) to be used during the screening process of the hybridomas was synthesized according to the active ester procedure using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrocloride (EDC) and *N*-hydroxysuccinimide (NHS) (*11*).

Immunization of Mice. Six BALB/c female mice (6 weeks old) were immunized according to the procedure described by Gonzalez et al. (*12*) with 20 μg (protein equivalents) of compound **3a** coupled to KLH (**3a**−KLH) in 100 μL of 0.1 M potassium phosphate buffer (PBS) [8 g/L of sodium chloride (NaCl), 0.2 g/L of potassium phosphate monobasic (KH_2PO_4), 1.2 g/L of sodium phosphate dibasic anhydrous (Na_2HPO_4), and 0.2 g/L of potassium chloride (KCl), pH 7.5] mixed 1:1 with Freund's complete adjuvant. After 4 weeks, each animal was boosted with an additional 20 μg using Freund's incomplete adjuvant, and serum was collected after 10 days. Antisera were screened for anti-molinate activity with the ELISA described below, and the mouse showing the highest anti-molinate activity received a third injection. Three days later, the spleen and the popliteal lymph nodes of the injected mouse were removed for hybridoma production.

Hybridoma Production and Selection. Hybridoma cell lines were produced by polyethylene glycol (PEG) fusion of spleen and lymph nodes cells with SP2/0 myeloma cells as described by Gonzalez et al. (*12*). Briefly, mouse spleen and lymph node cells were fused to murine myeloma cell line SP2/0 using a 50% PBS−PEG (MW 3000−3700) solution and dispensed into four 96-well microtiter culture plates in 100 μL of RPMI 1640 medium supplemented with 10 μg/mL gentamicin and 5% fetal calf serum (FCS). Hybridomas were selected in HAT medium, and 10 days after the fusion, cell-free culture supernatants were tested for the presence of anti-molinate antibodies by using a combination of noncompetitive and competitive ELISA. Well cultures giving a strong positive response in the noncompetitive ELISA and showing significant molinate recognition (at least 70% signal reduction in the competitive ELISA with 1 ppm molinate) were cloned by limiting dilution to ensure monoclonality.

Table 1. Molinate Competition Curve Parameters of the Tested Antibodies[a]

antibody	IC_{50} (ng/mL)	slope	range (ng/mL)	LOD[b] (ng/mL)	IC_{10}[c] (ng/mL)
pAb 245	211 ± 13	0.93 ± 0.20	40–1000	3 ± 1	24 ± 2
mAb 16C11	82 ± 2	0.99 ± 0.11	16–500	1 ± 1	7 ± 1
mAb 14D7	173 ± 8	1.12 ± 0.17	40–1000	3 ± 2	20 ± 1
mAb 5B7	∼20000				
mAb 12A4	∼2000				

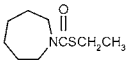
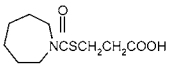
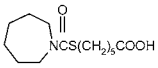
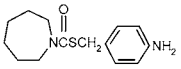
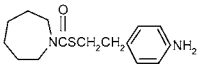
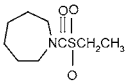
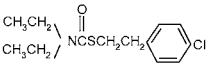
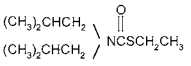
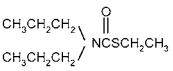
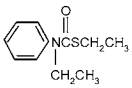
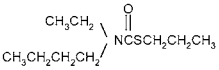
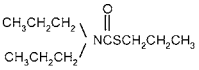
[a] All data are the mean ± SD of at least eight independent experiments. [b] LOD, limit of detection, defined as the concentration of the absorbance value equal to the absorbance at zero concentration minus 3 times the standard deviation. [c] IC_{10}, concentration that inhibits 10% the maximum signal.

Enzyme-Linked Immunoassay. *Noncompetitive Format.* Microtiter plates were coated with 0.2 μg/well of **3a**−OVA or **7b**−CONA in carbonate−bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were then blocked with OVA (5 mg/mL in 1× PBS), and 75 μL of the cell-free hybridoma supernatant was applied into each well. After a 1 h incubation period at room temperature, the plates were washed with 1× PBS containing 0.05% Tween 20 (PBST) three times, and then anti-mouse IgG conjugated to peroxidase, diluted 1/5000 with PBST, was added to each well. After a 1 h incubation period, the plates were washed and 100 μL of the peroxidase substrate (0.4 mL of a 6 mg/mL DMSO solution of 3,3′,5,5′-tetramethylbenzidine, 0.1 mL of 1% H_2O_2 in water in a total of 25 mL of 0.1 M citrate acetate buffer, pH 5.5) was dispensed into each well. The enzyme reaction was stopped after 30−40 min by the addition of 50 μL of 2 N H_2SO_4, and the absorbance was read at 450 nm in a microtiter plate reader (Multiskan MS, Labsystems).

Competitive Format. Prior to competition assays, the appropriate dilution of each hybridoma supernatant was determined under noncompetitive conditions to obtain a maximum absorbance close to 1.0 absorbance unit in the absence of molinate. The competition assay was performed as previously described (*10*). Briefly, 96-well microtiter plates were coated and washed as above, and then 50 μL/well of different molinate standards in PBST and 50 μL/well of antiserum or cell-free hybridoma supernatant of a predetermined concentration in PBST were applied. After a 1 h incubation period at room temperature, the plates were washed and developed as previously described.

Standard curves were normalized by expressing experimental absorbance values (B), as $(B/B_0) \times 100$, where B_0 is the absorbance value at zero molinate concentration. Absolute or normalized values were

Table 2. Cross-Reactivity of Some Thiocarbamates and Related Compounds[a]

	Compound		pAb 245		mAb 16C11		mAb 14D7		mAb 5B7		mAb 12A4	
			IC_{50} Mx10^{-7}	%CR	IC_{50} Mx10^{-7}	%CR	IC_{50} Mx10^{-7}	%CR	IC_{50} Mx10^{-7}	%CR	IC_{50} Mx10^{-7}	%CR
	molinate		12.4	100	4.7	100	9.3	100	139.0	100	107.0	100
A	3a		3.0	410	5.8	80	6.2	100	11.1	1250	19.4	550
A	3b		2.5	487	139.8	3	3.9	237	4.2	3300	27.5	389
A	7a		25.4	49		0	26.9	34	18.6	746	151.1	71
A	7b		10.3	120	66.7	7	27.1	34	3.3	4300	17.2	623
B	molinatesulfone		62.6	20		0		0		0		0
B	thiobencarb		1580	1		0	743.9	1		0	759.4	14
B	butylate		467	3		0	834.1	1	nd		nd	
B	EPTC		459	3		0	176.0	5	nd		nd	
B	cycloate		930	1		0	170.9	5	nd		nd	
B	pebulate		1716	1		0	213.3	4		0	1187	9
B	vernolate		408	3		0	258.6	4	nd		nd	

[a] Polystyrene 96-well microtiter plates were coated with 100 μL of 2 μg/mL **7b**–CONA. The appropriate dilutions of each antibody (Ab 245, 1/5000; Ab 16C11, 1/200; Ab 14D7, 1/400; Abs 12A4 and 5B7, 1/3000) were incubated with several concentrations of the compounds listed. The highest concentration used for compounds listed between molinate and **7b** was 2000 ng/mL and for those listed between molinatesulfone and vernolate was 100000 ng/mL. All data are the mean ± SD of at least three experiments unless otherwise noted. A value of 0 for % of cross-reactivity means that there was no inhibition at the highest concentration tested in each case. nd indicates that the specific compound–antibody pair was not tested.

fitted to a four-parameter logistic equation using Genesis Lite 3.03 [Life Sciences (U.K.) Ltd.] package software.

Cross-Reactivity. The selectivity of the selected monoclonal antibodies was characterized by determining the cross-reactivities with some thiocarbamates and other related compounds. Inhibition curves in a range from 0 to 100000 ng/mL were used in the competitive ELISA. After the data were normalized, the molar carbamate concentrations that caused 50% inhibition (IC_{50}) were used to calculate cross-reactivities according to the following equation:

$$\%\ \text{cross-reactivity} = 100 \times [IC_{50}(\text{molinate})/IC_{50}(\text{cross-reacting compound})]$$

Matrix Effects. *Solvent Tolerance.* Inhibition curves of molinate were prepared in PBS buffer (pH 7.5) containing 0, 1, 5, 12.5, and 25% (v/v) of acetonitrile or propylene glycol to determine the effect of organic solvent.

Ionic Strength. The effect of ionic strength on the performance of the monoclonal antibodies was evaluated by preparing molinate standard solutions in 0.1×, 1×, and 10× PBS. In addition, water samples from three different sources (tap water, surface water, and well water) were used to prepare standard curves.

RESULTS AND DISCUSSION

Hybridoma Production and Antibody Selection. After the cell-fusion process, a total of 44 hybridomas secreting antibodies that recognize the antigen were initially selected by screening against the immunizing hapten conjugated to ovalbumin (**3a**−OVA) and OVA. The specificity of these hybridomas to molinate was roughly evaluated in a competition assay. The molinate concentration (1000 ng/mL) used to compete for the antibody was the concentration of molinate that caused an 80% inhibition in the ELISA when pAb 245 was used (*10*). The competition assay was done in both ELISA formats, homologous (immunization antigen as coating) and heterologous (**7b**−CONA as coating antigen). Even though we were expecting the heterologous system to be more sensitive than the homologous

system (*13*), at this stage, the results appeared not to be dependent on the coating antigen, probably because the molinate concentration used in the inhibition assay was already high enough to produce an almost complete inhibition. The hybridomas tested in the assay showed diverse sensitivities for molinate, ranging from basically none to almost complete inhibition. To target the most sensitive antibodies, only the hybridomas showing >70% inhibition in both ELISA systems were selected and expanded for further studies. According to these criteria, eight hybridoma supernatants were selected and named: S5, S7, S8, S9, S12, S14, S16, and S20.

After the most sensitive hybridoma supernatants were identified, a more refined characterization was performed by determining the inhibition curves for the eight hybridoma supernatants. The working dilutions of the eight supernatants and the serum from the mouse used in the fusion process (M4) were determined on a checkerboard titration using **7b**−CONA-coated plates. They were defined as the dilution giving an absorbance value ≥0.8 in a noncompetitive ELISA. Hapten **7b** was chosen for coating as it was previously established to be the best coating hapten for pAb 245. Molinate inhibition curves were then performed using the eight supernatants, the mouse serum M4, and the rabbit polyclonal sera pAb 245.

Figure 1 shows all of the competition curves obtained. Compared to pAb 245 the mouse polyclonal serum M4 performance in the ELISA was very poor, with a very high IC_{50} value of ∼2000 ppb. However, with the hybridoma technique it was possible to identify and isolate those B-cells with the highest sensitivity for molinate. The potential of the hybridoma technique is clearly reflected by the data shown in **Figure 1**; the inhibition curves obtained with the different hybridoma supernatants showed substantially different characteristics. Most of the supernatants showed less sensitive curves than pAb 245, indicated by the fact that the IC_{50} values for them were 3−10-fold the IC_{50} value for pAb 245. This was true for supernatants 5, 7, 8, 9, 12, and 20 (**Figure 1**). On the other hand, the inhibition curve performed with supernatants 14 and 16 had lower IC_{50} values, which indicated that they were more sensitive than pAb 245. These two supernatants showed advantageous characteristics for the development of the ELISA for molinate. Therefore, they were cloned by limited dilution and retested for positives; this process resulted in the retrieval of the monoclonal antibodies mAbs 14D7 and 16C11. In addition, two clones from the low-sensitivity pool were also cloned by limited dilutions, resulting in the monoclonal antibodies mAbs 5B7 and 12A4.

Inhibition curves were performed with the four monoclonal antibodies (**Figure 2**). The competition curve obtained with the rabbit polyclonal sera was used as a reference showing an IC_{50} value of ∼211 ± 13 ppb. The minimum detectable amount of molinate (LOD), defined as the absorbance of the zero value minus 3 SD, was 3 ± 1 ppb, and the dynamic range of the assay extended from 40 to 1000 ppb (**Table 1**). MAb 14D7 IC_{50} was 172 ± 8 ppb, similar to that for pAb 245, with the advantage that mAb 14D7 showed a steeper slope (**Table 1**), which may provide a more accurate determination of molinate concentration. Interestingly, mAb 16C11 appeared to be the most sensitive antibody with the lowest IC_{50}, 82 ± 2 ppb, and the lowest limit of detection, 1 ± 1 ppb (**Table 1**).

Cross-Reactivity. To characterize the specificity of our monoclonal antibodies, several thiocarbamates and other pesticides were tested by competitive ELISA on **7b**−CONA-coated plates. The cross-reactivity measurements based upon molinate (100%) are shown in **Table 2**. The lower **B** panel shows the

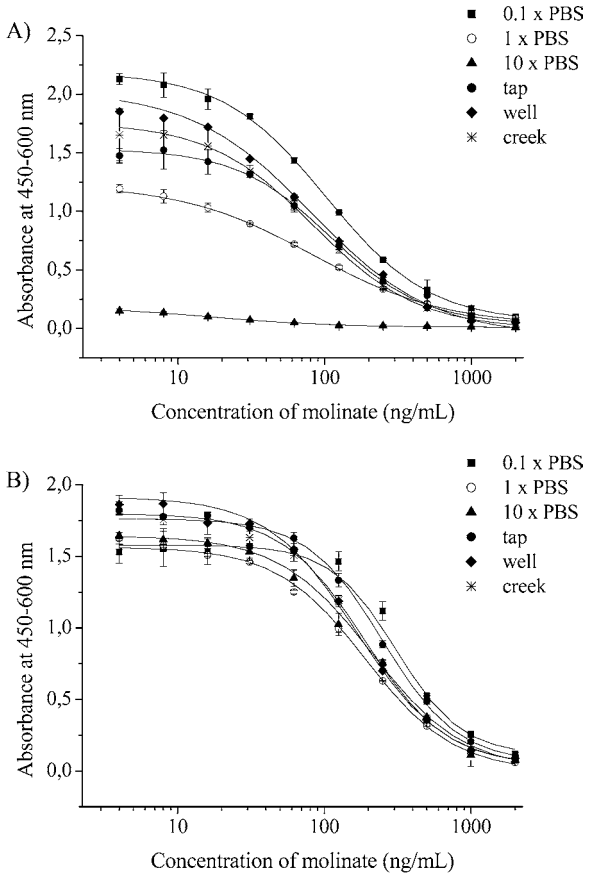


Figure 3. Effect of buffer ionic strength and matrix composition on the molinate standard curve for (**A**) mAb 16C11 and (**B**) mAb 14D7. Molinate standard solutions were prepared using the following systems: 10× PBS; 1× PBS; 0.1× PBS; tap water; well water; and creek water. Values are the mean of two independent experiments with three replicates each. IC_{50} values for each curve: **(A**, mAb 16C11) ■, 105 ng/mL; ○, 82 ng/mL; ▲, not applicable; ●, 108 ng/mL; ◆, 89 ng/mL; ∗, 85 ng/mL; **(B**, mAb 14D7) ■, 290 ng/mL; ○, 173 ng/mL; ▲, 210 ng/mL; ●, 240 ng/mL; ◆, 176 ng/mL; ∗, 190 ng/mL.

results of the cross-reactivity study with a series of other thiocarbamate pesticides and the molinate degradation product, molinate sulfone. None of the antibodies tested cross-reacted significantly (≤14%) with any of these compounds. These results indicate that none of these compounds will interfere, if present, in the assay.

The upper part of **Table 2**, panel **A**, shows the cross-reactivity pattern for the molinate haptens used during the assay development. Because hapten **3a** was used to raise the antibodies, the percent cross-reactivity for **3a** and its structural analogue **3b** were expected to be greater than that for molinate. This was true for all of the antibodies tested except for mAb 16C11, which showed only 3% of cross-reactivity with **3b** and a reactivity for **3a** equal to, or lower than, that for molinate (**Table 2**). The reactivity toward the coating hapten **7b** is of particular interest because the final sensitivity of the assay depends on the relationship between the sensitivity of the antibodies for molinate and for the coating hapten. To have a sensitive ELISA, the sensitivity of the antibody for molinate should be higher than that for the coating hapten. As expected, this was the case for mAbs 16C11 and 14D7, which had 7 and 40% of cross-reactivity, respectively. On the other hand, mAbs 5B7 and 12A4 had substantially higher sensitivity for **7b** than for molinate, which may explain why the IC_{50} values for these monoclonals are so high when **7b** is the coating hapten (**Table 2**). Overall,

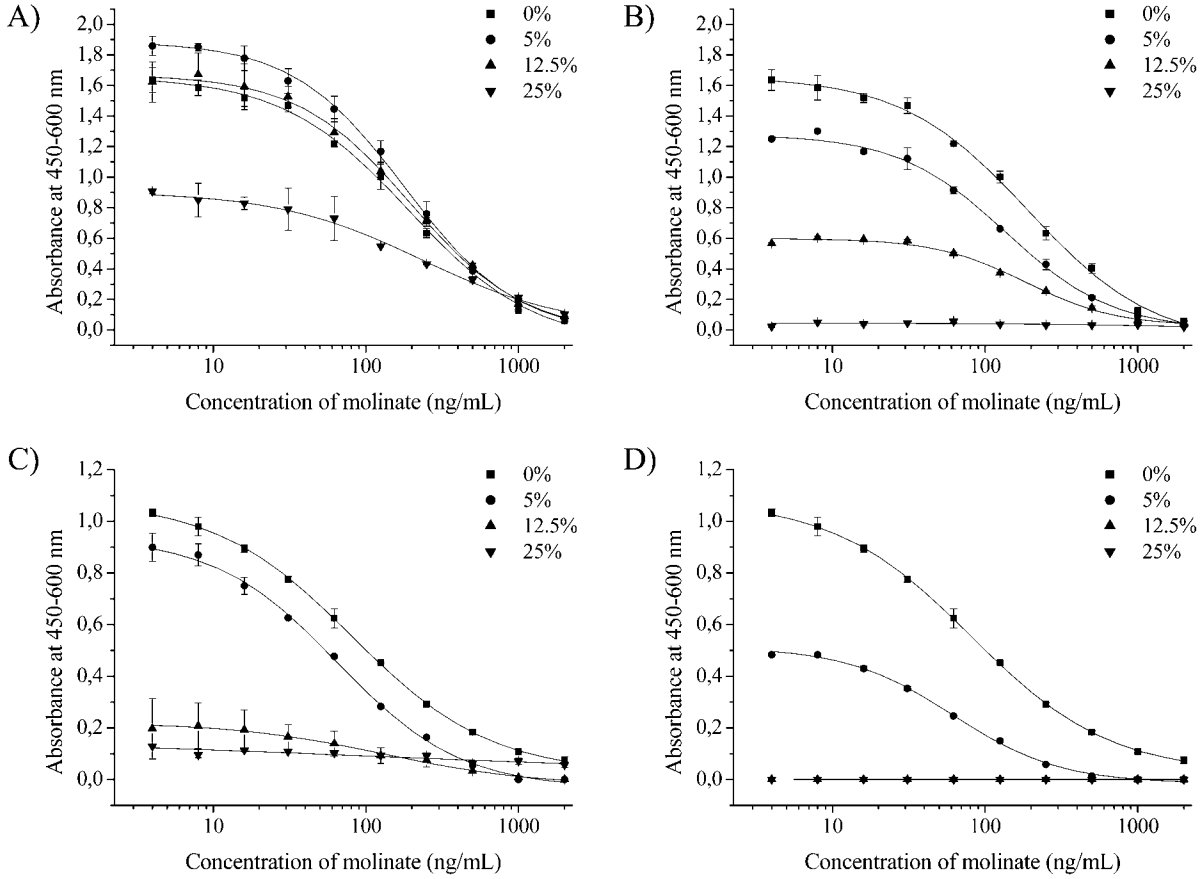


Figure 4. Mab 14D7 tolerance to the presence of organic solvent in the assay buffer as shown by the effect of propylene glycol or acetonitrile on the molinate standard curve for mAbs 16C11 and 14D7: **(A** and **B)** mAb 14D7 with different amounts of acetonitrile or propylene glycol, respectively; **(C** and **D)** mAb 16C11 with different amounts of acetonitrile or propylene glycol, respectively. Values are the mean of two independent experiments with three replicates each.

the analysis of the cross-reaction pattern for monoclonal antibodies indicated that the four monoclonals tested are molinate specific, but with differences in sensitivities. In addition, by analyzing the chemical structures of the compounds used in the assay, it appears that the monoclonal antibodies required the hexahydroazepine ring together with the carbothioate group for the antibody−antigen interaction. Finally, mAbs 14D7 and 16C11 appear to be very suitable antibodies, and they will be further tested.

Matrix Effect Studies. The evaluation of matrix effects is an important step in immunoassay development, in particular if the assay will be applied to samples with considerable variations in salt concentration. The effects of different kinds of matrices on the responses of mAbs 16C11 and 14D7 in the competition ELISA were tested to evaluate the ruggedness of the assay. For this purpose inhibition curves were prepared in buffer of different ionic strengths as well as in water samples from three different sources. Substantial differences were observed between mAb 16C11 and 14D7 performance as shown in **Figure 3**. The inhibition curve with antibody 14D7 was not severely altered by changes in ionic strength (**Figure 3B**) except for the 0.1× PBS buffer, which caused a 1.5-fold increase in the IC_{50} value. On the other hand, the mAb 16C11 inhibition curve dramatically changed. The absorbance readings increased, and the curve was shifted to the right at lower ionic strength (0.1× PBS), whereas at higher ionic strengths such as 10× PBS the absorbance readings are negligible (**Figure 3A**).

Additionally, the effect of different organic solvents was tested because organic solvents may be required to maintain the solubility of the analyte in aqueous solution. Polar (water miscible) solvents such as acetonitrile and propylene glycol are often used for this purpose in ELISA. **Figure 4** shows the effect of propylene glycol and acetonitrile on the molinate inhibition curves for mAbs 14D7 and 16C11. As shown in panel **C** of **Figure 4**, mAb 16C11 can tolerate only up to 5% of acetonitrile without compromising the sensitivity of the assay, whereas 5% of propylene glycol in the assay buffer starts to compromise the sensitivity by flattening the curve (**Figure 4D**; **Table 3**). On the other hand, mAb 14D7 can tolerate up to 12.5% of acetonitrile in the ELISA (**Figure 4A**; **Table 3**). Surprisingly,

Table 3. Effect of Organic Solvent in the Assay Buffer on the Monoclonal Antibody Inhibition Curves[a]

	mAb 14D7			mAb 16C11		
solvent	A_{max}	slope	IC_{50} (ng/mL)	A_{max}	slope	IC_{50} (ng/mL)
ACN						
0%	1.659	1.12	173 ± 8	1.081	0.99	82 ± 2
5%	1.888	1.18	181 ± 14	0.939	1.08	65 ± 4
12.5%	1.671	1.17	198 ± 14	0.223		
25%	0.906	0.90	237 ± 46	0.175		
PPG						
0%	1.659	1.12	173 ± 8	1.081	0.99	82 ± 2
5%	1.278	1.23	137 ± 10	0.514	1.24	62 ± 3
12.5%	0.599	1.47	184 ± 16			
25%	0.046					

[a] Assay conditions: coating antigen, **7b**–CONA (2 μg/mL); mAb 14D7 diluted 1:200; mAb 16C11 diluted 1:100 in 1× PBST; standard of molinate was prepared in PBST buffer containing different amounts of acetonitrile (ACN) or propylene glycol (PPG); anti-mouse IgG-HRP (1:5000). Values are the mean of two independent experiments with three replicates each.

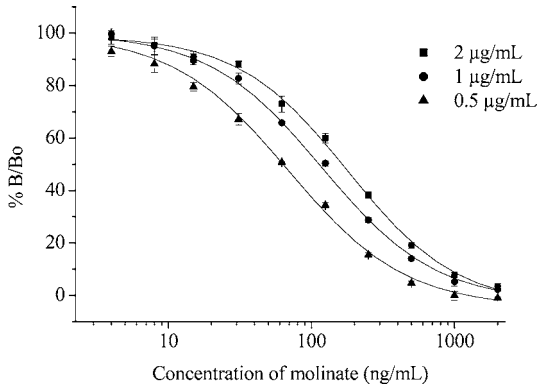


Figure 5. Mab 14D7 sensitivity increases as the amount of coating antigen decreases. Polystyrene 96-well microtiter plates were coated with 100 μL of 2, 1, and 0.5 μg/mL of **7b**–CONA. The inhibition assay was performed as described under Materials and Methods. Each point represents the mean ± SD of two independent experiments with three replicates.

Table 4. Decreasing the Coating Antigen Amount Increases the Sensitivity of the ELISA

coating antigen (μg/mL)	IC_{50} (ng/mL)	slope	A_{max}
2	172.9 ± 8.3	1.1 ± 0.1	1.66
1	118.5 ± 4.6	1.1 ± 0.0	1.54
0.5	69.2 ± 1.4	1.1 ± 0.0	0.96

5% of acetonitrile had a tendency to increase the absorbance, a phenomenon previously observed with polyclonal antisera (*10*). Propylene glycol at 5% did not affect the inhibition curve; however, at 12.5% and higher concentrations of this organic solvent the sensitivity was dramatically decreased (**Figure 4B**; **Table 3**).

In summary, mAb 14D7 appears to be a more suitable antibody than mAb 16C11 because it can function under more demanding conditions. Therefore, mAb 14D7 may be the antibody of choice because less sample manipulation will be required, resulting in a simpler and more rapid assay for environmental samples containing parts per billion levels of molinate. Moreover, the capability of mAb 14D7 to tolerate organic solvents in the assay will allow us to analyze samples containing low parts per billion levels of molinate by extraction of molinate with a highly volatile solvent, exchanging it into an ELISA compatible solvent.

MAb 14D7 Assay Optimization. On the basis of its high sensitivity for molinate and its ability to tolerate variations in the buffer assay, mAb 14D7 was chosen to further optimize the ELISA. By adjustment of assay conditions such as the amount of coating antigen, it was possibly to increase the sensitivity of the assay. **Figure 5** shows how the sensitivity of the assay, measured as the IC_{50}, increases as the amount of coating antigen on the microtiter plate decreases. The parameters of the inhibition curves are shown in **Table 4**. A decrease in the amount of coating antigen from 2 to 0.5 μg/mL causes the IC_{50} to shift from 172.9 ± 8.3 to 69.2 ± 1.4 ppb while conserving a steep slope of 1.1 ± 0.0 and a lower limit of detection of 2.5 ppb (**Table 4**). Further reduction in the coating antigen concentration causes a drop in the absorbance readings with no improvement in the assay sensitivity.

Conclusions. This work shows that sensitive and selective monoclonal antibodies can be developed for small compounds such as molinate through careful hybridoma screening. The careful development of mAb 14D7 has resulted in an immunoassay for molinate with an IC_{50} value at least 2-fold lower than the previously reported ELISA (*10*). The great advantage of the use of a monoclonal antibody is that it constitutes an unlimited source of a biologically defined reagent. This ELISA is specific for molinate over other thiocarbamate herbicides and can detect as little as 2.5 ppb of molinate. Considering that the maximum contaminant level in drinking water is 20 ppb (*14*), this new monoclonal-based ELISA could be a useful tool to monitor water quality.

In addition, mAb 14D7 will be a suitable tool to develop immunochromatographic cleanup or preconcentration columns, which will allow the analysis of samples containing molinate at the low parts per billion level.

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