Production of Monoclonal Antibodies to the *N*-Methylcarbamate Pesticide Propoxur

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Monoclonal antibodies (MAbs) to the pesticide propoxur were produced from haptens with carboxylic spacer arms of different lengths introduced at the carbamate group of the analyte structure. MAbs were subsequently characterized in the conjugate-coated format using these immunizing haptens and newly synthesized compounds as homologous and heterologous assay conjugates, respectively. Appropriate combinations of immunoreagents resulted in competitive enzyme immunoassays (ELISA) with I_{50} values in the low nanomolar range (6.5−17.9 nM). A modification of the conjugate-coated format consisting of the simultaneous incubation of the MAb and the peroxidase-labeled secondary antibody in the presence of the analyte resulted in an assay with an I_{50} value of 4.4 nM. This one-step conjugate-coated ELISA format is as simple and fast as the antibody-coated format but without the need of synthesizing enzyme−hapten conjugates. Major *N*-methylcarbamate pesticides were not recognized by the MAb. This immunoassay should reasonably allow the rapid, low-cost, and sensitive determination of propoxur in food, soils, and the environment at levels of regulatory and practical importance.

Keywords: *ELISA; immunoassay; insecticide; monoclonal antibodies; hapten design; hapten heterology; ELISA format; carbamate*

INTRODUCTION

Propoxur (2-isopropoxyphenyl methylcarbamate), which is most commonly sold under the trade name Baygon, is a nonsystemic *N*-methylcarbamate pesticide with contact and stomach action. It has a rapid knockdown effect on insects and a long residual activity. Due to its low toxicity to mammals and other vertebrates, propoxur is an economically important insecticide manufactured in very large quantities, and it is widely used to control agricultural and household insect pests (Tomlin, 1997; Wang et al., 1998).

Propoxur, like most *N*-methylcarbamates, is thermally unstable and decomposes to the phenol under the usual gas chromatography conditions (Liška and Slobodník, 1996). On the basis of the pioneering work by Moye et al. (1977), further modified for use on food samples by Krause (1985), HPLC with postcolumn derivatization and fluorescence detection is the preferred technique for the sensitive and selective determination of *N*-methylcarbamate pesticides in environmental waters and food (EPA Method 531.1) (McGarvey, 1993; Yang et al., 1996). However, this method presents some limitations derived from the fact that laborious cleanup, concentration, and derivatization steps are needed to achieve the desired sensitivity. Thus, the method requires sophisticated equipment that is not available in most analytical laboratories, and it is not very well suited for the analysis of a large number of samples. In fact, most pesticide regulatory programs analyze for *N*-methylcarbamates only a part of the total number of food samples analyzed for other active ingredients, such as organophosphorus pesticides (Gunderson, 1995).

Immunoassays are analytical methods based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are simple, cost-effective, and field-portable, do not require sophisticated instrumentation, and are able to analyze many samples simultaneously. All of these features, along with the large number of pesticides for which antibodies have been produced over the past few years, have promoted the acceptance of immunochemical techniques among analytical chemists as alternative and/or complementary methods for the analysis of agrochemicals (Hammock and Gee, 1995; Meulenberg et al., 1995; Dankwardt and Hock, 1997). This is particularly true for pesticides that are difficult and/or costly to determine due to their physicochemical characteristics and for large monitoring programs. In these situations, the use of immunoassays as a screening technique could provide a great saving of resources, effort, and toxic solvents.

The aim of this work was to obtain high-affinity monoclonal antibodies to propoxur in order to develop sensitive enzyme immunoassays as an alternative to liquid chromatographic techniques for the screening of large numbers of agricultural and environmental samples.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Pesticide and metabolite standards were from Riedel-de Haën (Seelze, Germany) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in dry *N*,*N*-dimethylformamide (DMF) and stored at 4 °C. Starting products for

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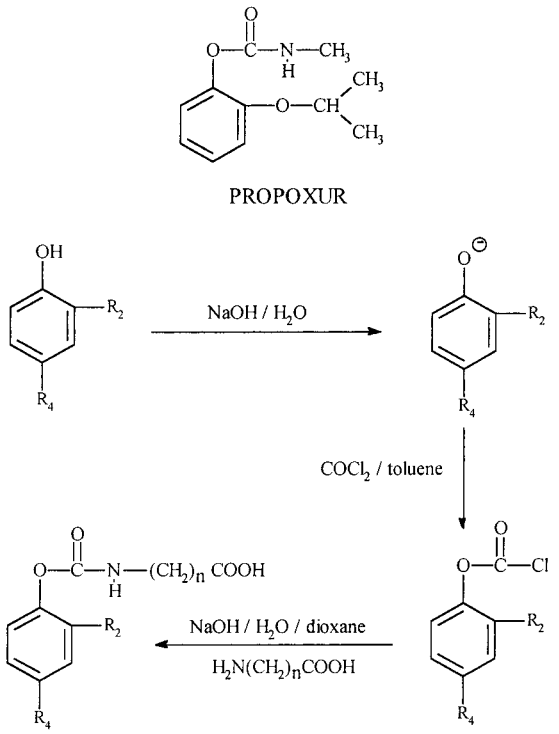
10.1021/jf0009596 CCC: $20.00
Published on Web 12/07/2000



hapten	n	R_2	R_4
PRNP	2	$OCH(CH_3)_2$	H
PRNB	3	$OCH(CH_3)_2$	H
PRNH	5	$OCH(CH_3)_2$	H
OEPR	5	OCH_2CH_3	H
OIPR	5	$CH(CH_3)_2$	H
PIPR	5	H	$CH(CH_3)_2$
OMPR	5	OCH_3	H
PMPR	5	H	OCH_3
OCPR	5	CH_3	H
PCPR	5	H	CH_3
PNH	5	H	H

Figure 1. Reaction scheme of the synthesis of haptens that preserve the carbamate group.

the synthesis of haptens and hapten−protein coupling reagents were obtained from Fluka-Aldrich Química (Madrid, Spain).

Ovalbumin (OVA), Freund's adjuvants, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin fraction V (BSA), hybridoma fusion and cloning supplement (HFCS), and polyethylene glycol (PEG) 1500 were purchased from Boehringer Mannheim (Barcelona, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins (referred to as secondary antibody) were obtained from Dako (Glostrup, Denmark). Culture media (high-glucose Dulbecco's Modified Eagle's medium with GLUTAMAX I and sodium pyruvate, DMEM), fetal calf serum (Myoclone Super Plus), and supplements were from Gibco BRL (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). P3-X63-Ag8.653 mouse plasmacytoma line was from American Tissue Type Culture Collection (Rockville, MD).

Polystyrene culture plates (high-binding plates, catalog no. 3590) were from Costar (Cambridge, MA). Plates were washed with a 96PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria). Absorbance in the ELISA wells was read in dual-wavelength mode (490−650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 300 spectrometer (Sunnyvale, CA), operating at 300 MHz for ^{1}H. Chemical shifts are reported relative to tetramethylsilane. Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Hapten Synthesis. Propoxur haptens used in this work were prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, at the hydroxyl group of the phenolic precursor, either after being reacted with phosgene to form the carbamate group (Figure 1) or directly by O-alkylation (Figure 2). Haptens were characterized by ^{1}H NMR (Table 1).

6-[[(2-Isopropoxyphenyloxy)carbonyl]amino]hexanoic Acid (PRNH, Figure 1). 2-Isopropoxyphenol (12.6 mL, 85.2 mmol) was added to 50 mL of 2.5 M sodium hydroxide. Thereafter, a slight excess of phosgene (50 mL of a 20% phosgene solution in toluene, 96.6 mmol) was slowly added, and the reaction was incubated at room temperature for 4 h with magnetic stirring (WARNING: phosgene is a highly toxic gas. Work in a well-ventilated fume hood and handle carefully.) After the addition

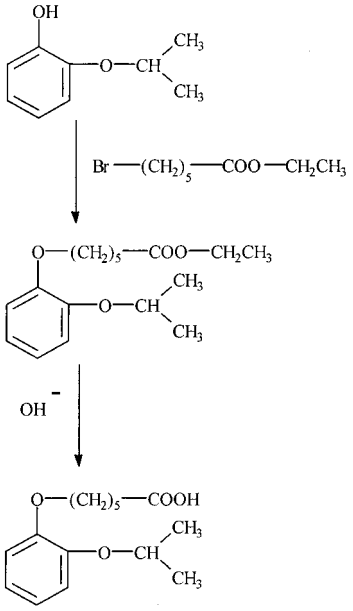


Figure 2. Reaction scheme of the synthesis of the PROH hapten.

of ethyl acetate, the organic phase was dried over anhydrous sodium sulfate and evaporated to dryness at reduced pressure. On the basis of its behavior on gas chromatography, the 66.7% of the resulting brown solid was assumed to be 2-isopropoxyphenyl chloroformate (10.2 g) and subsequently used without further purification. Aminohexanoic acid (3.0 g, 22.6 mmol) was dissolved in 4 mL of 4 M sodium hydroxide, and the solution was cooled at 4 °C. The crude chloroformate (2.43 g, 11.3 mmol) was dissolved in 4 mL of cold 1,4-dioxane, and this solution, along with 6 mL of cold 4 M sodium hydroxide, was added to the amino acid solution in five equal portions, with at least 5 min being allowed between additions. The reaction mixture was stirred in an ice bath for 1.5 h. After acidification to pH 4.0 with concentrated hydrochloric acid, the carboxylic derivative, which separated as an oil, was extracted with ethyl acetate (three 35-mL portions). The ethyl acetate

Table 1. ^{1}H NMR Spectral Data for Haptens

	δ values[a]								
	aromatic substituent						spacer arm		
hapten	$OCH(CH_3)_2$	CH_2CH_3	$CH(CH_3)_2$	OCH_3	CH_3	ArH	NCH_2	$(CH_2)_x$[b]	CH_2COOH
PRNP	4.56(m), 1.27(d)					6.86−7.16(m)	3.46(q)		2.62(t)
PRNB	4.56(m), 1.23(d)					6.85−7.15(m)	3.26(q)	1.89(m)	2.43(t)
PRNH	4.55(m), 1.27(d)					6.86−7.15(m)	3.20(q)	1.40−1.70(m)	2.32(t)
OEPR		4.05(q), 1.39(m)				6.89−7.14(m)	3.26(q)	1.37−1.70(m)	2.36(t)
OIPR			3.27(m), 1.21(d)			7.03−7.31(m)	3.11(q)	1.40−1.71(m)	2.37(t)
PIPR			3.21(m), 1.22(d)			7.00−7.23(m)	2.90(q)	1.39−1.67(m)	2.32(t)
OMPR				3.84		6.90−7.20(m)	3.26(q)	1.40−1.70(m)	2.36(t)
PMPR				3.77		6.87−7.04(m)	3.20(q)	1.41−1.66(m)	2.31(t)
OCPR					2.21	7.04−7.26(m)	3.27(q)	1.39−1.70(m)	2.37(t)
PCPR					2.32	6.97−7.15(m)	3.25(q)	1.41−1.70(m)	2.37(t)
PNH						7.08−7.37(m)	3.20(q)	1.38−1.65(m)	2.30(t)
PROH	4.51(m), 1.30(d)					6.84−7.01(m)	4.02(t)[c]	1.55−1.85(m)	2.36(t)

[a] ^{1}H NMR spectra were obtained with a spectrometer operating at 300 MHz. Samples were dissolved in acetone-d_6, and chemical shifts are reported in parts per million relative to tetramethylsilane. [b] The number of methylene groups in the central part of the spacer arm was three for all of the haptens except for PRNB, for which it was one. [c] This methylene group in the PROH hapten should be noted as OCH_2.

phase was washed several times with diluted hydrochloric acid and extracted with 1 M bicarbonate solution (three 50-mL portions). After the solution had been cooled in an ice bath, it was acidified again with concentrated hydrochloric acid. The precipitated product was collected, washed with water, and dried to yield 4.0 g of crude PRNH. It was then crystallized from hexane/ethyl acetate (70:30) to yield 790.0 mg of the hapten.

The rest of the haptens shown in Figure 1 were synthesized in the same way by either using a different amino acid (PRNP and PRNB haptens) or replacing the phenolic precursor by a different one (OEPR, OIPR, PIPR, OMPR, PMPR, OCPR, PCPR, and PNH haptens).

6-(2-Isopropylphenyloxy)hexanoic Acid (PROH, Figure 2). To 50 mL of dry acetone were added stoichiometric amounts (20 mmol) of 2-isopropoxyphenol, potassium carbonate, and ethyl 6-bromohexanoate. After reflux for 12 h, the mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in 50 mL of ethyl acetate, washed with water (2 × 50 mL), 1 M NaOH (3 × 50 mL), and 4 M NaCl (2 × 50 mL), and finally dried over anhydrous sodium sulfate. After evaporation of the solvent, 50 mL of 1 M NaOH was added to the residue, and the solution was stirred while heated under reflux for 1.5 h. The solution was then acidified with concentrated hydrochloric acid and extracted with ethyl acetate (2 × 50 mL). The organic phase was evaporated, and the solid obtained (2.63 g) was subsequently crystallized from hexane/ethyl acetate (70:30) to obtain 1.22 g of the hapten.

Preparation of Immunizing Conjugates. Haptens PRNP, PRNB, and PRNH were covalently attached to BSA using the modified active ester method (Langone and Van Vunakis, 1982). Twenty-five micromoles of the hapten was incubated overnight at room temperature with stoichiometric amounts of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in 0.5 mL of DMF. After centrifugation, 400 μL of the clear supernatant containing the active ester was slowly added to 2 mL of a 15 mg/mL BSA solution in 50 mM carbonate buffer, pH 9.6. The mixture was allowed to react at room temperature for 4 h with stirring, and finally the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, pH 7.4, as eluant. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between the carrier protein and conjugates in the region of maximum absorbance of haptens. The hapten to protein molar ratio of conjugates was then estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. By assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, apparent molar ratios were estimated as 13, 14, and 14 for haptens PRNP, PRNB, and PRNH, respectively.

Preparation of Coating Conjugates. All of the haptens were covalently attached to OVA using the mixed-anhydride method (Rajkowski et al., 1977). Eighteen micromoles of the hapten was allowed to react at room temperature for 1 h with stoichiometric amounts of tri-*n*-butylamine and isobutyl chloroformate in 200 μL of DMF. One hundred microliters of the resulting activated hapten was added to 30 mg of OVA in 2 mL of 50 mM carbonate buffer, pH 9.6. The coupling reaction was incubated at room temperature for 2−3 h with stirring, and the conjugates obtained were purified as described for the immunogens. The extent of coupling of each hapten to OVA was determined by UV spectrophotometry. By assuming additive absorbance values, hapten to protein molar ratios were evaluated as 4, 5, 5, 5, 6, 4, 4, 8, 2, 10, 1, and 2 for haptens PRNP, PRNB, PRNH, PROH, PMPR, OMPR, PCPR, OCPR, OEPR, OIPR, PIPR, and PNH, respectively.

Production of MAbs to Propoxur. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA-PRNP, -PRNB, and -PRNH conjugates. First dose consisted of 30 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. Two subsequent injections were given at 3-week intervals emulsified in incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection in adjuvant, mice received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski et al. (1979). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at a density of $\sim 2 \times 10^5$ cells/well in 100 μL of s-DMEM. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) containing 2% HFCS (v/v) was added to each well.

Hybridoma Selection and Cloning. Eight to 11 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized propoxur. The screening consisted of the simultaneous performance of a noncompetitive and a competitive indirect enzyme-linked immunosorbent assay (ELISA), to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and to recognize propoxur, respectively. For each culture supernatant, the signal obtained in noncompetitive conditions was compared with the competitive one, and the ratio of both absorbances was used as the criterion for selecting high-affinity antibody-secreting clones. Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 2% HFCS (v/v) as a cell growth promoter instead of the classical feeder layer of

thymocytes and peritoneal macrophages. Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of MAbs. Antibodies were purified directly from late stationary phase culture supernatants by saline precipitation with saturated ammonium sulfate followed by affinity chromatography on a HiTrap Protein G column (Pharmacia Biotech AB, Uppsala, Sweden). Most culture supernatants were able to provide enough MAb (5−10 mg/100 mL) for characterization studies and further work. Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

Competitive ELISAs. Flat-bottom polystyrene ELISA plates were coated by adding 100 μL/well of the OVA−hapten conjugate solution in 50 mM carbonate buffer, pH 9.6. The optimal concentration for each conjugate was previously determined by checkerboard titration. After overnight incubation at room temperature, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Standards (50 μL/well) and antibody (50 μL/well) at the apropriate concentration were added. Standards were prepared in assay buffer (100 mM sodium phosphate, 137 mM NaCl, pH 7.2) by serial dilutions from a stock solution in DMF, using borosilicate glass tubes, and the antibody solution was prepared in assay buffer containing 0.1% BSA and 0.02% thimerosal. Plates were incubated for 1 h and washed as before. Then, 100 μL/well of a 1:2000 dilution of the secondary antibody in PBST (PBS containing 0.05% Tween 20) was added, incubated for 1 h, and washed again. Finally, peroxidase activity bound to the wells was determined by adding 100 μL/well of the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.4). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance at 490 nm was read. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation using Sigmaplot (Jandel Scientific) and Softmax (Molecular Devices, Sunnyvale, CA) software packages.

RESULTS AND DISCUSSION

Production of Antibodies to Propoxur. Propoxur, like most pesticides, is a small and simple organic molecule non immunogenic by itself and lacking a functional group for coupling to proteins. Therefore, the synthesis of haptens resembling as much as possible the structure and electronic distribution of propoxur is a necessary and critical step in the production of high-affinity antibodies. Accordingly, three hapten were synthesized (PRNP, PRNB, and PRNH) by the introduction of a spacer arm of different lengths through the carbamate group characteristic of this pesticide (Figure 1). In this way, that is, by elongation of an aliphatic chain already present in the analyte, both the structure and the electronic distribution of the propoxur molecule are hardly modified, as demostrated by the high-affinity MAbs previously obtained following this chemical approach for carbaryl (Abad et al., 1997), methiocarb (Abad et al., 1998), and carbofuran (Abad et al., 1999).

Three cell fusion experiments were performed with each of the BSA conjugates of the immunizing haptens. In the screening of culture supernatants only wells showing a signal inhibition higher than 80% in the presence of 1 μM propoxur with respect to that obtained in the absence of analyte were considered as containing high-affinity antibody-secreting clones. Although all of the cell fusions rendered wells with antibodies recognizing the homologous coating conjugates, only four wells fulfilled the above-mentioned criterion, so they were accordingly cloned and expanded. Two of these hybridomas came from the PRNP hapten, while the other two came from the PRNB hapten.

Characterization of the MAbs. The ability of the MAbs to recognize propoxur was first estimated from inhibition curves in the conjugate-coated ELISA format using the three immunizing haptens as assay conjugates. Under these conditions, I_{50} values in the low nanomolar range were obtained. No significant differences in the affinity to propoxur with respect to the spacer arm length of the assay hapten were found. Therefore, of the three immunizing haptens, only PRNH was used as the common homologous assay hapten for further work.

At this point, several heterologous haptens were also synthesized to try to improve the affinity of the antibodies to propoxur. The PROH hapten was synthesized by direct O-alkylation of 2-isopropoxyphenol (Figure 2), so the only relevant difference with respect to the immunizing haptens is the absence of the carbamate group. The rest of the haptens were obtained by a chemical route identical to that used for the immunizing haptens but starting from different phenolic precursors. As a consequence, these heterologous haptens, although preserving the carbamate group, differ in both the chemical group present in the aromatic ring and the ring position substituted (Figure 1).

Prior to competitive experiments, the ability of the antibodies to recognize the heterologous conjugates was evaluated by checkerboard titration. All of the MAbs recognized the conjugate based on the PROH hapten, probably as a consequence of its strong resemblance to the immunizing haptens. With respect to the hapten series with modifications in the ring substituents, none of the MAbs was able to recognize coating conjugates based on haptens with a chemical group at the 4-position (PIPR, PMPR, and PCPR). On the contrary, haptens with a chemical group at the 2-position were recognized by the MAbs to a greater or lesser extent, depending on the chemical nature of the substituent. Thus, the OEPR hapten, which bears an ethoxy group at the 2-position, was recognized by all of the MAbs, and the OIPR and OMPR haptens, with an isopropyl and a methoxy group, respectively, were recognized by three MAbs. Even the OCPR hapten, with only a methyl group at this position, was recognized by one antibody (LIB-PRNB33). However, the PNH hapten, which does not have any ring substituent because it was derived from phenol, was not recognized by any of the MAbs. Therefore, a substituent must be present in heterologous haptens at the same ring position as in the immunizing haptens to be properly recognized by the MAbs, although modifications in the chemical group attached to the aromatic ring are tolerated.

Inhibition curves using propoxur as competitor were performed with the four selected MAbs in combination with all of the coating conjugates that were recognized by each antibody. I_{50} values for each of these combinations are shown in Table 2. The effect of hapten heterology on the affinity to propoxur depended on both the antibody and the conjugate under consideration. In this respect, the I_{50} value obtained with the PROH hapten was 25 times higher than that obtained with the homologous hapten for LIB-PRNP21 MAb (I_{50} = 456.0 nM with OVA-PROH versus I_{50} = 17.9 nM with OVA-PRNH). Nevertheless, the use of PROH reduced to the half the I_{50} value obtained with the homologous hapten for LIB-PRNB33 MAb (I_{50} = 7.4 nM with OVA-PROH versus I_{50} = 18.2 nM with OVA-PRNH). With respect to the antibodies, only LIB-PRNB33 MAb experienced

Table 2. Propoxur I_{50} Values (Nanomolar) of the MAbs Obtained, Determined in the Conjugate-Coated ELISA Format[a]

coating	LIB-PRNP15	LIB-PRNP21	LIB-PRNB21	LIB-PRNB33
OVA-PRNH	8.7	17.9	25.6	18.2
OVA-PROH	35.2	456.0	33.8	7.4
OVA-OEPR	7.1	16.2	30.2	9.7
OVA-OIPR	7.9		24.0	6.5
OVA-OMPR	16.6		16.8	10.8
OVA-OCPR				10.1

[a] The concentrations of conjugate and antibody used in these competitive assays were those previously determined as optimal by checkerboard titration for each combination of immunoreagents.

a noticeable increase in the affinity to propoxur as a consequence of the use of heterologous coating conjugates (I_{50} = 6.5 nM with OVA-OIPR versus I_{50} = 18.2 nM with OVA-PRNH). The antibodies showing the highest affinity to propoxur were LIB-PRNP15 and LIB-PRNB33, so they were chosen for further work.

Evaluation of the One-Step Conjugate-Coated Format. Apart from the conjugate-coated format, another very frequently used ELISA configuration is the so-called antibody-coated or enzyme−tracer format. In this format the analyte and an enzyme−hapten conjugate compete for the binding sites of the antibody, which is immobilized to the plate, either directly or through a capture protein. After incubation, the enzymatic activity bound to the wells is determined by adding the adequate substrate, as it is the case for the conjugate-coated format. The main advantage of this configuration is that, unlike the conjugate-coated format, the assay takes place in only one step, which increases the simplicity and reduces the assay time and the number of washing steps. These characteristics have made this format the configuration of choice for commercial ELISA kits. However, the enzymes used in the preparation of the conjugates (usually horseradish peroxidase or alkaline phosphatase) are expensive, and immobilized antibodies frequently fail to properly recognize enzyme tracers. Moreover, at least in our experience, some enzyme tracers require special storage conditions to maintain their activity in the long term, which may be a problem under field conditions, during transport, and for laboratories that are not used to this technology.

We decided to assess whether a simple approach consisting of the simultaneous incubation of the anti-propoxur MAb and the secondary antibody in the presence of the analyte could result in a sensitive ELISA for propoxur, thus putting together the main advantages of the conjugate-coated and the antibody-coated ELISA formats. To this purpose, slight modifications of the ELISA procedure described under Materials and Methods were introduced. Following the addition of 50 μL/well of standards, 50 μL/well of a mixture of the MAb and the secondary antibody was added, instead of adding and incubating the individual immunoreagents separately. To reduce the background signal to acceptable levels (<0.1), the immunoreagent mixture was prepared in assay buffer containing 0.01% Tween 20 instead of 0.1% BSA. Moreover, to obtain a higher maximum signal, the secondary antibody was used at a final 1:1000 dilution instead of the usual 1:2000 dilution. I_{50} values from competitive curves obtained with the LIB-PRNP15 and LIB-PRNB33 MAbs using this approach are shown in Table 3. With respect to the standard conjugate-coated ELISA format, the affinity

Table 3. Propoxur I_{50} Values (Nanomolar) of the Best MAbs Obtained, Determined in the One-Step Conjugate-Coated ELISA Format[a]

coating	LIB-PRNP15	LIB-PRNB33
OVA-PRNH	53.1	15.9
OVA-PROH	53.4	8.4
OVA-OEPR	36.1	13.0
OVA-OIPR	37.2	10.4
OVA-OMPR	28.7	24.3
OVA-OCPR		6.2

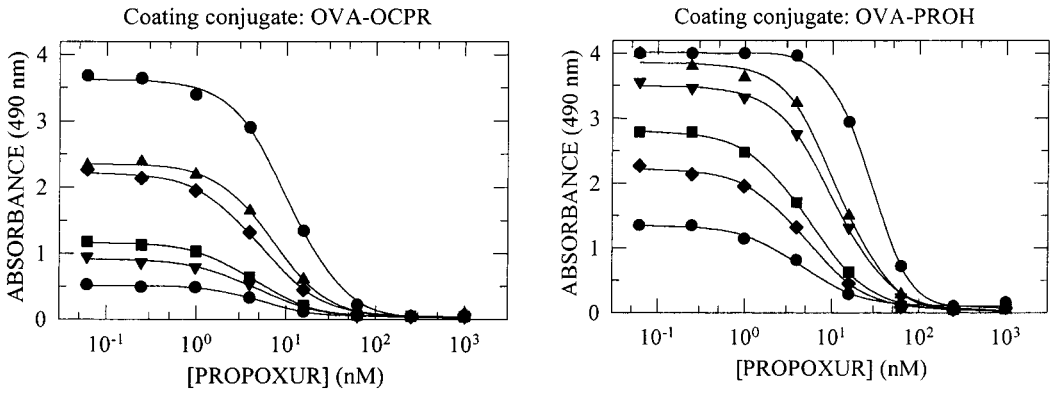
[a] The concentrations of conjugate and antibody used in these competitive assays were those previously determined as optimal by checkerboard titration for each combination of immunoreagents.

to propoxur of LIB-PRNP15 MAb decreased for all of the coating conjugates (higher I_{50} values), whereas very similar I_{50} values were found for LIB-PRNB33 MAb. Further work with this antibody in combination with the two best coating conjugates (OVA-PROH and OVA-OCPR) revealed that both haptens were equally suitable for the development of an immunoassay for propoxur (Figure 3). Taking into account both the sensitivity and the reagent consumption, the best combinations were OVA-OCPR at 1 μg/mL with LIB-PRNB33 at 0.1 μg/mL (A_{max} = 1.17; I_{50} = 4.34 nM) and OVA-PROH at 1 μg/mL with LIB-PRNB33 at 0.05 μg/mL (A_{max} = 1.35; I_{50} = 4.43 nM). Therefore, although identical sensitivities were obtained with both conjugates, OVA-PROH is preferable to OVA-OCPR because a slightly higher maximum absorbance was obtained with a lower antibody concentration.

Cross-Reactivity Studies. The specificity of the LIB-PRNB33 MAb was evaluated in the one-step conjugate-coated format using OVA-PROH as the immobilized conjugate. Competitive assays with the propoxur metabolite propoxur-hydroxy and the most important *N*-methylcarbamate pesticides were performed, and the obtained I_{50} values were used to calculate cross-reactivities. As shown in Figure 4, the assay was very specific for propoxur, because the highest cross-reactivity value was 1.4% for bendiocarb. Therefore, significant interferences derived from the presence of other *N*-methylcarbamates or the product of the metabolic transformation of propoxur should not be expected in the application of this immunoassay to agricultural or environmental samples.

CONCLUSIONS

The application of a hapten synthesis strategy previously used for other *N*-methylcarbamates has resulted in the obtention of a panel of MAbs highly specific to propoxur with sensitivities in the low nanomolar range. In addition, the antibodies herein described constitute to our knowledge the first ones reported for this insecticide. With the idea in mind of a further improvement of assay sensitivity with respect to that achieved using homologous coating conjugates, nine haptens with modifications in the ring sustituents or in the spacer arm were synthesized and evaluated as heterologous conjugates. Although hapten heterology was not in this case as decisive as in previously reported examples for other analytes, this strategy in fact allowed a 3-fold improvement in the sensitivity of LIB-PRNB33 MAb for propoxur. Finally, the usual procedure of the conjugate-coated ELISA format was modified to decrease the total assay time and to simplify the method. This goal was simply achieved by simultaneously adding the MAb, the



			curve parameters				
[coating conjugate] (μg/mL)		[MAb] (μg/mL)	A_{max}	B	I_{50}	A_{min}	r^2
OVA-OCPR	1.0	0.05	0.51	1.54	5.24	0.04	0.998
	1.0	**0.1**	**1.17**	**1.32**	**4.34**	**0.04**	**1.000**
	1.0	0.3	2.36	1.39	7.02	0.02	0.999
	2.0	0.05	0.92	1.23	4.62	0.03	0.997
	2.0	0.1	2.23	1.26	4.99	0.03	0.999
	2.0	0.3	3.63	1.38	10.42	0.01	0.999
OVA-PROH	**1.0**	**0.05**	**1.35**	**1.29**	**4.43**	**0.10**	**0.995**
	1.0	0.1	2.81	1.23	5.42	0.02	0.999
	1.0	0.3	3.86	1.46	11.43	0.01	0.998
	2.0	0.05	1.98	1.21	6.13	0.01	1.000
	2.0	0.1	3.51	1.35	10.24	0.02	0.999
	2.0	0.3	4.02	1.90	26.49	0.06	1.00

Figure 3. Competitive checkerboard titration in the one-step conjugate-coated ELISA format of the LIB-PRNB33 MAb in combination with OVA-PROH and OVA-OCPR coating conjugates. Experimental points were fitted to the sigmoidal equation $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the asymptotic maximum absorbance (A_{max}), B is the slope at the inflection point, C is the concentration value in nanomolar at the inflection point (I_{50}), and D is the asymptotic minimum absorbance (A_{min}). Best combinations of immunoreagents are indicated in boldface print.

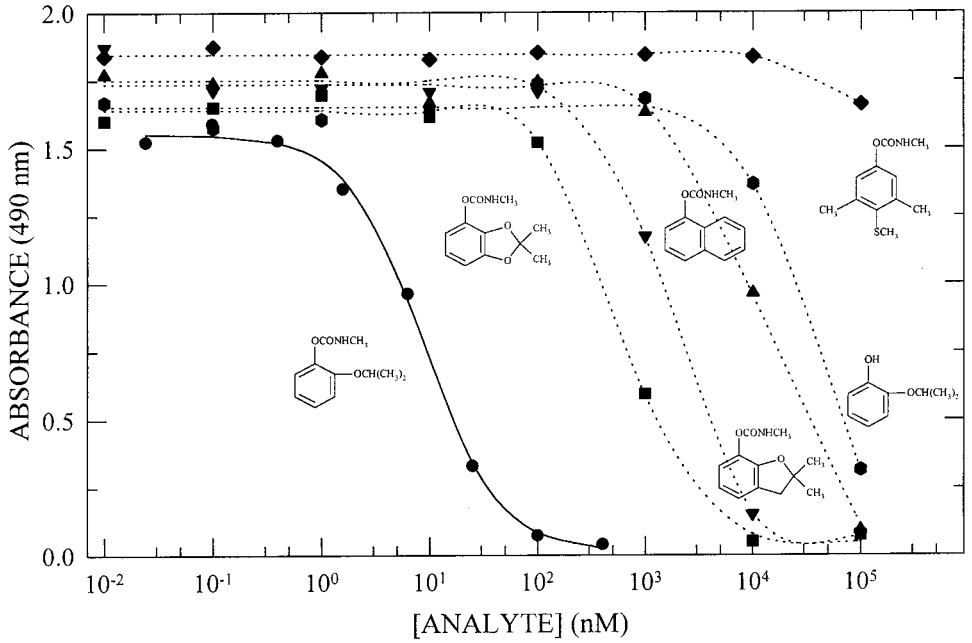


Figure 4. Specificity of the propoxur immunoassay. ELISA plates were coated with the OVA-PROH conjugate at 1 μg/mL, and the LIB-PRNB33 MAb was used at 0.1 μg/mL. Cross-reactivity values, calculated as (I_{50} of propoxur/I_{50} of other compound) × 100, were as follows: propoxur, 100.0% (●); bendiocarb, 1.4% (■); carbofuran, 0.6% (▼); carbaryl, 0.1% (▲); propoxur-hydroxy, 0.1% (⬢); methiocarb, 0.01% (◆).

secondary antibody, and the analyte to precoated wells. In our oppinion, this approach bears all of the advantages of the direct assays but avoids the need of synthesizing labile enzyme−hapten conjugates. Using

this new ELISA format, the best combination of immunoreagents provided an I_{50} value of 4.4 nM (0.72 ppb). The limit of detection (LOD) and the limit of quantitation (LOQ) of this assay, estimated from the I_{90} and I_{80} values for standards in buffer, were 0.18 and 0.32 ppb, respectively. Taking into account these parameters, the usual sample dilution requirements of immunoassays in order to minimize matrix effects, and the common procedure for extracting *N*-methylcarbamates from fruits and vegetables (De Kok and Hiemstra, 1992), this ELISA should reasonably allow the determination of propoxur at 15−20 ppb in real samples. It should be noted that this value is only slightly higher than the LOQ for propoxur of the conventional chromatographic technique (5−10 ppb), albeit the immunoassay is a simpler technique with a higher sample throughput. Therefore, this monoclonal immunoassay seems to be a very promising analytical technique for cost- and labor-effective monitoring of propoxur in environmental and agricultural samples, for which maximum residue limits are in the 0.05−3.0 ppm range, depending on the crop (*Codex Alimentarius*, 1999).

ABBREVIATIONS USED

BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HFCS, hybridoma fusion and cloning supplement; I_x, analyte concentration reducing the assay signal to *X*% of the maximum value; LOD, limit of detection; LOQ, limit of quantitation; MAb, monoclonal antibody; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; PEG, polyethylene glycol; UV−vis, ultraviolet−visible.

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Received for review July 31, 2000. Revised manuscript received September 16, 2000. Accepted October 16, 2000. This work was supported by a grant (ALI96-1232) from the Comisión Interministerial de Ciencia y Tecnología (Spain).

JF0009596