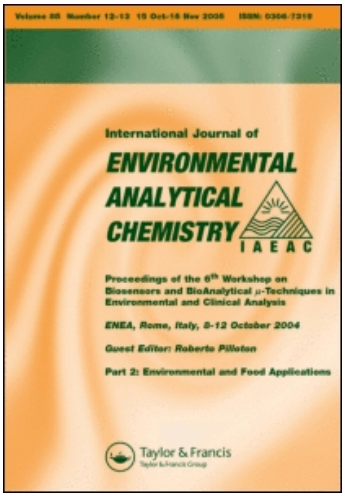

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Ligand Choice Strategy for the Purification of Polyclonal Antibodies Used for Improved Immunochemical-Based Analytical Methods for the Herbicide Isoproturon

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LIGAND CHOICE STRATEGY FOR THE PURIFICATION OF POLYCLONAL ANTIBODIES USED FOR IMPROVED IMMUNOCHEMICAL-BASED ANALYTICAL METHODS FOR THE HERBICIDE ISOPROTURON

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A common problem in developing immunochemical-based analytical methods is that the desired antibodies represent only a small portion of the heterogeneous antibody population. Affinity purification of polyclonal serum requires the correct choice of ligands used to extract antibodies featuring similar characteristics in their recognition properties (similar paratop group and dissociation constants). This paper describes several strategies of ligand design and synthesis used to purify polyclonal anti-serum generated against the herbicide isoproturon. A synthetic ligand mimicking the analyte isoproturon produced a 2.6 fold increase in isoproturon antibody concentration compared to an extraction using an IgG specific commercial gel. Another strategy involving the use of herbicide-protein conjugates resulted in affinity gels efficient in extracting 6 times more specific antibodies than the IgG commercial gel. The performances of immunochemical-based analytical methods using these refined antibodies were significantly improved. An indirect enzyme immunoassay gave an IC_{90} of 0.01 ng/mL, when assayed with river water samples and a better correlation with HPLC analyses was also reached (r = 0.998) for those samples. Immobilization of such antibodies on activated silica afforded a high capacity

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immunoaffinity column, which retained an average of 8.2 μg of isoproturon for 20 mg of immobilized antibodies per gram of silica.

Keywords: Affinity chromatography; antibody; purification; isoproturon; enzyme immunoassay; immunoaffinity chromatography

INTRODUCTION

Immunochemical methods have been widely employed in environmental analytical applications. Pesticides have been a primary focus of these applications[1] and a number of immunoassays have been developed as screening methods. The rapid growth in using these techniques is mainly attributed to, amongst other things, the availability of polyclonal and monoclonal antibodies, directed against more than 100 pesticides, which were developed over the last 15 years.[2] Increasing attempts toward the development of monoclonal antibodies have been reported in order to overcome the difficulties encountered when polyclonal anti-serum is used for analytical applications. Polyclonal antibodies have recognition sites for different portions of the antigen molecule and a wide distribution of association constants for each site. As a consequence of the presence of high affinity antibodies, harsh conditions may be needed to elute all the bound analyte in immunoaffinity columns, which compromises their further use. Furthermore a substantial fraction of the analyte may become irreversibly bound and therefore unrecoverable after the first percolation on a virgin column. Similar problems are observed when immunoassays are developed, affecting the precision and accuracy of this technique.

However, it is possible to avoid most of these difficulties by adopting the appropriate method for antibody purification. Affinity chromatography purification allows a selection of a given portion of polyclonal antibodies. A more homogeneous fraction is obtained for antibodies recovered using the same eluting conditions.

Our laboratories have been involved in developing antibodies targeting isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea], a major representative of the phenylurea herbicides, widely used in Europe. These antibodies were used to prepare immunoaffinity columns for sample preparation purposes.[3,4] An enzyme immunoassay was also developed using the same antibodies and reached the detection limit of the European guideline of 0.1 μg/L.[5] However, problems were encountered while developing these applications, mainly due to the heterogeneity of the polyclonal anti-serum. First, the immunosorbent we produced exhibited a low capacity, limiting the development of an on-line automated immuno-preconcentration, which requires a minimum amount of solid

sorbent with a high binding capacity. Also, a standardized procedure was difficult to set up as problems with elution were observed from column to column. Second, the enzyme immunoassay produced excessive recoveries compared to results obtained when the same surface water samples were processed by HPLC with a prior clean-up step using an immunoaffinity column. A high day to day RSD (>10%) was also observed. In both these applications, the antibodies were used, either directly from the crude serum (enzyme immunoassay) or after a classical purification using a commercially available gel to extract the IgG fraction.

This paper describes several attempts at isoproturon antibody purification using affinity chromatography. The main challenge was first to design the appropriate ligand to be immobilized on a solid support, to permit the specific adsorption of the target antibodies, and second to set up the operating conditions to elute the retained antibodies without altering their activity.

EXPERIMENTAL SECTION

Apparatus

Fractions of effluent from the affinity columns used for antibody purification were monitored by measuring the absorbance at 280 nm with a UVICON 860 spectrophotometer. A BioRad microplate washer was used for microtiter plate washing. Optical densities of microtiter wells were measured on a dual beam Titertek multiscan MCC with a 492 nm sample filter and 620 nm reference filter. Data were transmitted to a spread sheet program for analysis. The instrument was checked periodically by a spectrocheck plate and software (QC Technology, New-York). HPLC analyses were performed using a Millenium 2010 workstation (version 2.10) including a Waters 600 controller solvent delivery system, a Waters 712 autosampler and a Waters 486 absorbance detector set to 244 nm. The analytical column was a Waters Symmetry C18, 5 μ, 100 Å, HPLC cartridge (15 cm $\times$ 0.39 cm i.d). Melting points were determined with a Mettler FP 62 apparatus. NMR spectra were measured on a Bruker 200 MHz spectrometer. Chemical shift values are given in ppm downfield from internal tetramethylsilane.

Materials

Anti-isoproturon antibodies were obtained by immunizing 2 New-Zealand rabbits with BSA-isoproturon immunogen.[5] The IgG was isolated by affinity chromatography using Avidchrom® gel purchased from Unisyn Technologies

(Hopkinton, MA, USA). Sepharose® CL-4B was supplied by Pharmacia. Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Holland). Ovalbumin, bovine β-lactoglobulin (βLG), goat anti-rabbit IgG peroxidase conjugate (second antibody), 1,1′-carbonyldiimidazole-activated agarose, Tween 20 and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma. Dot blots were performed on nitrocellulose membranes supplied by Schleicher & Schuell (product number 439 194). LC grade water was prepared by purifying demineralized water in a Milli-Q (MQ) filtration system (Millipore, Bedford, MA). Antibody solutions were concentrated using Amicon Centriplus 30 (exclusion size of 30kD) ultrafiltration units. Dialysis tubing (10 mm in diameter with a 12 000-14 000 molecular weight cut off) was purchased from Spectrum Medical Industries Inc. (Los-Angeles, CA). Flat bottom polystyrene microtiter plates were obtained from Dynatech laboratories, Inc. (Chantilly, VA). The sorbent used to prepare immunoaffinity columns consisted of 40 μm glutaraldehyde activated silica beads, with a pore size of 275 Å and was a kind gift of JT-Baker B.V. (Netherlands). Pesticide standards (isoproturon and other related compounds) were obtained from the pesticide repository of the Food Research Division and were stated to be at least 99% pure by the respective manufacturers. Other chemicals were from Prolabo, Merck, or Fluka.

Buffers

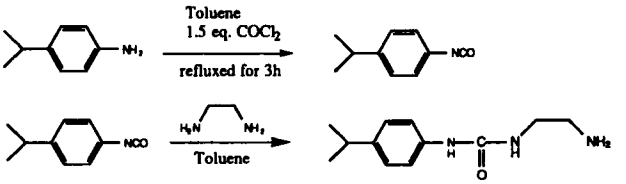
Phosphate-Buffered Saline (PBS, adjusted to pH 7.4) contained 20 mmol of NaH_2PO_4 and 140 mmol of NaCl per liter of deionized water. PBS-BR was obtained by adding 1.5% (w/v) of skimmed milk as a blocking reagent in PBS. Washing buffer (PBS-T) consisted of 0.1% Tween 20 (v/v) in PBS. Tris Buffered Saline (TBS, pH 7.5) contained 50 mmol of Tris, 150 mmol of NaCl per liter of deionized water. TBS-T consisted of 0.1% Tween 20 (v/v) in TBS. Citrate buffer (pH 5.0) consisted of 51 mmol of Na_2HPO_4 and 24 mmol of citric acid per liter of deionized water. The substrate used for the dot blot test consisted of 5 mg of 4 chloro-naphthol dissolved in 30 mL of MeOH/TBS (10/90 v/v) and 60 μL of 30% H_2O_2. The substrate for the titer determination consisted of 17.5 mg OPD and 10 μL of 30% H_2O_2 in 25 mL of citrate buffer. Coating buffer (pH 9.6) contained 13 mmol of Na_2CO_3 and 35 mmol of $NaHCO_3$ per liter of deionized water.

Affinity Ligand Synthesis

Figure 1 shows the synthesis path of isoproturon derivatives used as affinity ligands. Ligand (I), which kept the urea moiety, was obtained according to the

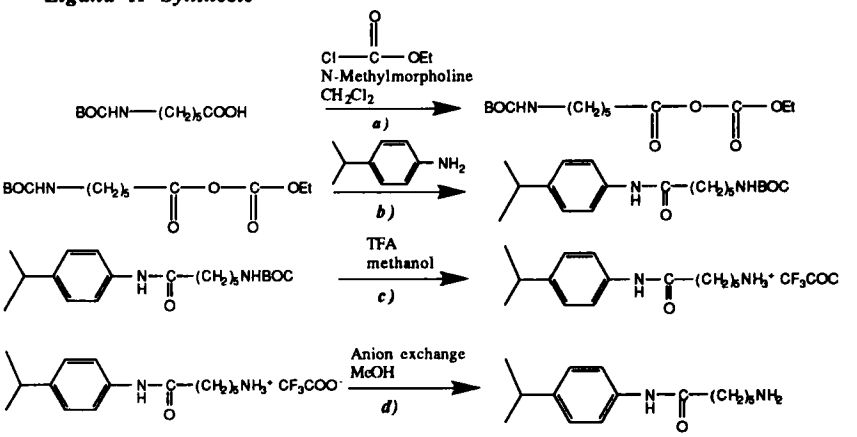
following description. A solution of 7 g (51 mmol) of 4-isopropylaniline in dry toluene (40 mL for 5 mL of aniline derivative) was cooled in an ice and water mixture before the addition of an excess of phosgene $COCl_2$ (80 mmol for 40 mmole of aniline derivative), with stirring. Thirty milliliters of toluene were added to the mixture which was stirred at room temperature overnight, then gently refluxed for two hours. The white solid (the remaining aniline hydrochloride) was eliminated by filtration and toluene was evaporated. The viscous isocyanate intermediate was dissolved in toluene and added dropwise to an ethylenediamine solution (130 mmol). The resulting solution was stirred overnight at room temperature, filtered, extracted with methylene chloride. The organic solution was washed three times with 30 mL aliquots of 1M HCl and saturated Sodium carbonate. The organic layer was dried over $MgSO_4$ and the

Ligand I Synthesis



Ligand I

Ligand II Synthesis



Ligand II

FIGURE 1 Synthesis strategies of ligands to be used in isoproturon antibody purification.

solvent was evaporated to give yellow crystals. The reaction yield was 32%; mp 198°C; ^{1}H NMR (DMSO-d_6) δ 8.47 (s, 1 H, Ar-N*H*), 7.34 (d, J = 2.1 Hz, 2 H, Ar-$H_{3,5}$), 7.1 (d, J = 2.0 Hz, 2 H, Ar-$H_{2,6}$), 6.1 (s, 1 H, N*H*CO), 3.45 (s, 2 H, NH_2), 2.73 (m, 1 H, C*H*$(CH_3)_2$), 2.3 (t, 2 H, -CH_2CH_2-) 1.18 (d, J = 7.5 Hz, 6 H, (CH_3)$_2$CH); ^{13}C NMR (DMSO-d_6) δ 155.7 (1C, C=O), 141.3 (1C, Ar-iPr), 138.5 (1C, Ar-NH), 126.56, 125.34, 120.69, 118.2 (4C, Ar-$C_{2,3,4,5}$), 32.9 (1C, -NH-*C*H_2), 33.16 (1C, -*C*H$(CH_3)_2$).

Ligand (II) was obtained according to a two step procedure (Figure 1). The first step consisted in protecting the amine function of 6-aminocaproic acid using a conventional N-Boc protection.[6] A 10 M NaOH solution (4.6 mL) was added to 6-aminocaproic acid (6.1 g, 46.5 mmol) dissolved in dioxan/water (60/30). The resulting solution was cooled in an ice and water mixture before the addition of di-tert-butyl dicarbonate (Boc_2O) (11.6 g, 1.1 eq.) dissolved in dioxane, with stirring. The mixture was stirred at room temperature overnight. Thirty milliliters of ethyl acetate were then added to the solution which was acidified with $KHSO_4$ (pH 2-3). The aqueous solution was extracted with ethyl acetate then the organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. N-terbutoxycarbonyl aminocaproic acid crystallized progressively as a white solid. The reaction yield was 60%; ^{1}H NMR ($CDCl_3$) δ 4.6 (t, 1 H, BocN*H*), 3.1 (m, 2 H, Boc-NH-CH_2), 2.3 (t, 2 H, -CH_2COOH), 1.8, ..., 1.3 (m, 6 H, -CH_2-(CH_2)$_3$-CH_2-), 1.4 (s, 9 H, Boc); ^{13}C NMR ($CDCl_3$) δ 178.8 (1C, COOH), 156 (1C, -O-CO-NH), 79.1 (1C, $(CH_3)_3$*C*-OCONH)), 40.2 (1C, -NH-*C*H_2), 33.8, ..., 24.2 (7C, aliphatic chain), 28.3 (3C, (*C*H_3)$_3$C-).

The second step consisted in activating the carboxylic acid function of N-terbutoxycarbonyl 6-aminocaproic acid before its addition to isopropylaniline according to the following procedure. A solution of Boc 6-aminocaproic acid (4 g, 17 mmol) in methylene chloride (45 mL) was added to 1.9 mL of N-methyl morpholine and cooled to −10°C in an ice and 1M NaCl mixture. Ethyl chloroformate (1 eq., 1.88 g) was added to the resulting solution with gentle stirring for 30 minutes, after which a solution containing 2.1 g isopropylaniline (0.9 eq., 2.1 mL) and 1.9 N-methyl morpholine in 31 mL methylene chloride was added. The mixture was kept at a temperature below 0°C for the first 3 hours then was stirred at room temperature for 12 hours. The solution was extracted with methylene chloride and the organic layer washed successively with 0.03 M HCl and saturated Na_2CO_3. The resulting solution was dried over $MgSO_4$ and evaporated to dryness. The crude residue was purified by flash chromatography with AcOEt:CH_2Cl_2/30:70 as eluent to give white crystals. The liberation of the amine function was achieved by dissolving the solid in MeOH/TFA (Trifluoroacetic acid): 10/1 and stirring the solution for 30 minutes. The solvent was evaporated and the residue was crystallyzed in diethyl ether. The white

precipitate formed, was filtered and washed with diethyl ether. The reaction yield was 30%. The recovered amine salt was passed through an anion exchange column using methanol as the eluent. The solvent was evaporated to give a yellow oily compound. NMR data for the ionized compound: ^{1}H NMR (DMSO-d_6) δ 7.9 (s, 3 H, NH_3^+), 7.5 (d, 2 H, Ar-$H_{3,5}$), 7.1 (d, 2 H, Ar-$H_{2,6}$), 2.8 (q, 2 H, -CH_2-NH_3^+), 2.73 (m, 1 H, $CH(CH_3)_2$), 2.3 (t, 2H, -CH_2-CONH-), 1.7,..., 1.2 (m, 6H, -$(CH_2)_3$-), 1.2 (d, 6H, $(CH_3)_2$CH-); ^{13}C NMR (DMSO-d_6) δ 171.1 (1C, CONH), 143.2 (1C, Ar-iPr), 137.4 (1C, Ar-NH), 126.56, 119.4 (4C, Ar-$C_{2,3,4,5}$), 36.3 (1C, -NH-CH_2), 33.1 (1C, -$CH(CH_3)_2$), 27.1, ..., 24.1 (4C, aliphatic chain).

Protein Conjugates as Affinity Ligands

The carboxylic acid derivative of isoproturon (IPCOOH) and chlortoluron (CLTCOOH) (Figure 2) were prepared according to a previously described procedure.[5] These compounds were conjugated to β lactoglobulin (βLG) via the mixed anhydride method[7] using only 27 molar equivalents of the anhydride-activated herbicide derivative in dioxane. The recovered protein was dialyzed against water then freeze-dried and stored at 4°C. The molar ratio of pesticide per carrier protein was calculated by determining the free amino groups of the lysine side chains with 2,4,6-trinitro-benzenesulfonic acid (TNBS) reagent.[8] The differential absorption of the native and the conjugated proteins at 420 nm allowed the determination of an average conjugation ratio.

Affinity Gel Synthesis and Use

1,1′-carbonyldiimidazole agarose was used for ligand (I) and (II) immobilization. Each ligand was dissolved in DMSO (300 mg) before addition to 5 mL of agarose washed with cool water (4°C). The coupling reaction was maintained under a mild stirring at 4°C for 12 hours, before treating and packing the resulting affinity columns. Sepharose® CL-4B (10 mL gel suspension) was used for protein conjugate immobilization and was activated according to the cyanogen bromide method.[9] The conjugated protein linked to the IPCOOH or CLTCOOH derivative was added to the coupling buffer (0.2M Na_2CO_3) at a ratio of 100 mg/10mL of gel (concentration of 10 mg/mL) and maintained under agitation for 12 hours at 4°C. All the modified gels were then washed 3 times with the corresponding coupling buffer to recover the unbound ligands, 5 times with water and 0.1 M phosphate buffer (pH 7.2) containing 0.1 M NaCl. The amount of unbound protein-conjugate ligand was determined by direct absorbance reading at 280 nm and by BCA protein assay. A small quantity of

protein conjugated beads was washed and hydrolyzed in a 1 M NaOH solution for 72 hours with mixing. The amount of incorporated protein was then estimated in the filtrate using a BCA protein assay. It was, however, difficult to set up a reliable technique allowing the determination of the amount of bound ligand (I) and ligand (II).

The modified agarose (5 mL of swollen gel in 0.1 M phosphate buffered saline, pH 7.2) was packed in a column (1 cm diameter) and washed with 5 volumes of 0.1 M PBS. One fifth diluted crude anti-serum or IgG (5 mg/mL) was percolated through the column at the rate of 0.5 mL/min and the effluent was kept at 4°C. Unbound antibodies were removed by washing with 50 mL of PBS. Then, elution was performed with the following eluents (80 mL): (a) 0.1M Phosphate buffer (pH 7.2) containing 1 M NaCl, (b) 0.05 M acetate buffer (pH 2.8), (c) 0.1 M PBS

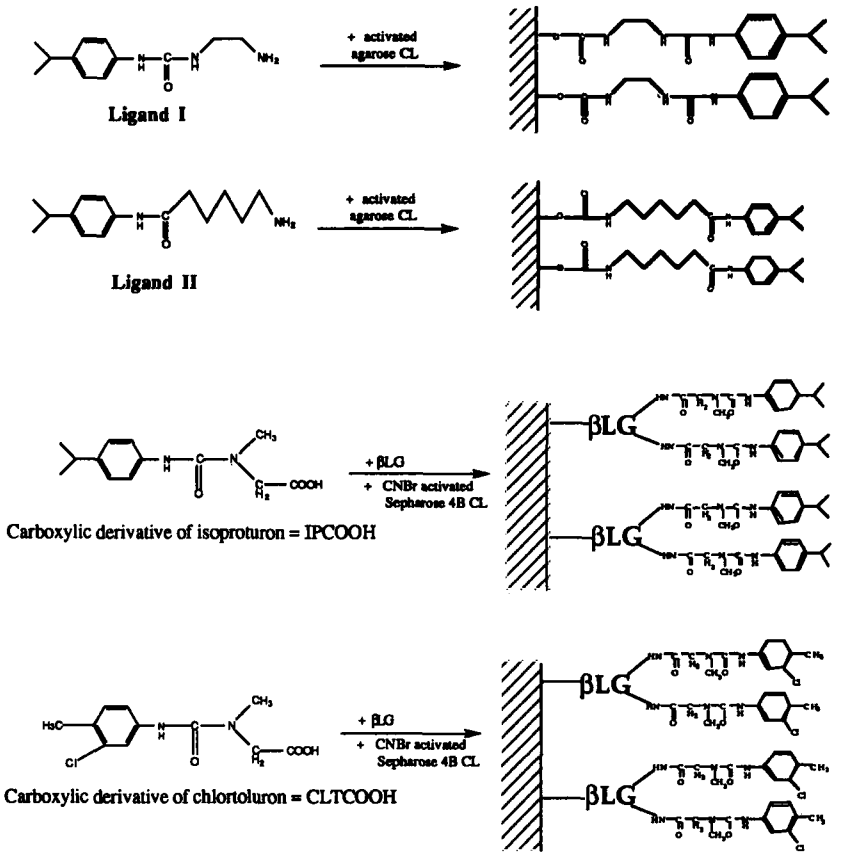


FIGURE 2 Ligands used for the design of affinity chromatography gels to purify anti-isoproturon antibodies

buffer containing varying amounts of ethylene glycol, (d) isoproturon or its carboxylic acid derivative dissolved in PBS (2% MeOH) at various concentrations, (e) pesticide solution containing various amounts of ethylene glycol. The eluted fractions were concentrated on Centriplus ultrafiltration modules and then dialyzed against 0.9% NaCl. Extensive dialysis was necessary to ensure complete antibody-antigen dissociation. All the recovered fractions were tested for the presence of antibodies by antibody dot blot according to the procedure described below. The purified antibodies were stored at 4°C prior to immobilization on the silica solid support or to their use in enzyme immunoassay. IgG pure fractions obtained by a preliminary step using Avidchrom® purification gel, were percolated on the affinity gels instead of crude anti-serum so as to ensure a reliable estimation of the recovery yield related to the use of each eluting agent.

Test for Presence of Isoproturon Specific Antibodies

(Dot Blot and ELISA): Two microliters of isoproturon-conjugated βLG (βLG-IP) (5 mg/mL) were blotted on a nitrocellulose membrane. The strips were blocked for one hour in PBS-BR prior to the incubation of the purified antibody solution (25 μg of proteins, 2 hour incubation at room temperature). After 3 washing steps with 10 mL of PBS-T, 1/2000 diluted peroxidase conjugated goat anti-rabbit antibodies were added to the strips for a 2 hour incubation. The membranes were then washed 3 times with 10 mL of TBS-T and finally put in the substrate solution (4-chloro-naphthol). The intensity of the colour spot that appeared on the strip was proportional to the amount of anti-isoproturon antibodies in the test solution. The titer of the IgG fraction and that of affinity purified ones were also compared using 1 μg/mL βLG-IP coated microtiter plates.[10] The antibody starting solutions were at 1 mg/mL. The procedure was maintained as described in a previous work.[5]

FPLC Analyses

The effluent fractions were analyzed using a fast protein liquid chromatography system. The Solutions (100 μL at 1 mg/mL) were applied to a MonoQ column in 20 mM ethanolamine/HCl, (pH 9) at the rate of 0.5 mL/min and then eluted with 0-1 M NaCl gradient (45 minutes). Proteins were monitored by their absorbance at 280 nm. Dot Blot tests were performed for all fractions.

Isoproturon Immunoaffinity Column Preparation and Capacity Evaluation

The aldehyde groups (glutaraldehyde) on the activated silica matrix were allowed to covalently link to the amine function of the purified antibodies (either IgG or affinity purified IgG) by a reductive amination. The resulting immunosorbent (0.5 g packed into a 3 mL plastic syringe barrel) was first conditioned with 10 mL of PBS and then with 5 mL of LC-grade water. To study the change of capacity of the different immunosorbents, 50 mL of spiked sample at 200 μg/L with isoproturon (10 μg) was percolated through the cartridge. The study of breakthrough volume of isoproturon was allowed by percolating serial volumes (50, 100, 150, 200, 300, 350, 500 mL) of solutions containing 50 ng of isoproturon. The column was then washed with 8 mL of LC-grade water. The elution was performed with 8 mL of a 70% MeOH/30% LC-grade water solution. The recovered isoproturon solution was evaporated to 250-500 μL and an aliquot of 50 μL was injected into the L.C. system. Isoproturon was separated using the following linear gradient: 40%-53% acetonitrile in water from 0 to 15 min, 53%-100% from 15 to 20 min.

Competitive Immunoassay

The indirect competitive ELISA procedure was similar to that reported by Newsome et al.[10] A 1 mL aliquot of crude anti-serum diluted 1/15,625 in PBS-BR or of refined antibodies (100 ng/mL) was added to 25 μL of spiked MQ water or river water sample. After mixing and incubation at 4°C for an hour, 200 μL was added to the wells of the sensitized plate in triplicate. The addition of the second antibody and the plate reading was achieved as described previously.[5] The standard curves consisted of 16 concentrations of isoproturon (0, 0.015-150 ng/mL) in MQ water and filtered river water. The antibody cross-reactivity was also determined using the same procedure with 16 concentrations of each tested pesticide.

RESULTS AND DISCUSSION

Different approaches have been described in literature for the design of affinity ligands useful in antibody purification. Proteins have been widely employed to isolate sub-groups of antibodies. Protein A and protein G are the most common examples of proteic ligands devoted to IgG extraction from crude anti-sera. Several attempts were reported to search for low-molecular weight ligands capable of performing or mimicking the selectivity of such complex immuno-

globulin binding proteins as protein A. Porath and co-workers[11-13] developed a novel and rapid method for selectively purifying antibodies using a structurally simple ligand with a molecular weight of less than 400. To purify antibodies based on the specificity of the variable fragment, two approaches have been reported Horstman et al.[14] immobilized the same hapten used for immunization directly on Sepharose®4B in order to purify anti-paraquat monoclonal antibodies from ascites fluid, which resulted in developing a rapid and efficient one-step purification procedure. The second approach was reported by Assil et al[15] who used a protein conjugate of the target analyte as a ligand. This resulted in extracting IgG that recognized only the molecule attached to the carrier protein. The advantage of this method is that it allows the derivatization of Sepharose® in a buffer medium. Furthermore, a recent study demonstrated that a longer spacer arm improves the antibody-antigen interaction.[16] A protein being a flexible spacer, may enhance this effect, if non-specific interactions are not observed.

Chemistry and Preparation of Affinity Ligands

In the present work, protein conjugates as well as low molecular weight ligands were assayed. The design of the synthetic ligands was aimed at mimicking the analyte, isoproturon. It also took into account the necessity of providing an appropriate available function for a further coupling to a functionalized solid support and easiness of the synthesis procedure. Figure 1 shows isoproturon mimicking derivatives and their synthetic pathway. Ligand (I) kept a urea function and incorporated two methylene moieties as a spacer arm. A synthesis difficulty was however encountered. It was difficult to avoid a second addition of the left amine function on another molecule of isopropyl-phenylisocyanate. This could be limited by operating under an excess of ethylenediamine (5 eq.) and by a slow addition of the isocyanate intermediate. Ligand (II) did not present synthesis difficulties and incorporated a longer spacer arm of 5 methylene moieties. The urea function was replaced by an amide group, but the substituted aromatic ring was kept intact. Both ligands incorporated a terminal amine function to be used for covalent attachment to a carbonyl diimidazole activated agarose CL, which is compatible with a coupling in an organic solvent medium.

Protein conjugates, used as affinity ligands, were obtained by linking β lactoglobulin (βLG) to anhydride activated carboxylic derivatives of phenylurea herbicides. βLG was chosen as the carrier protein since it showed no cross-reactivity with the anti-serum. Two proteic ligands were synthesized incorporating isoproturon and chlortoluron derivatives (Figure 2). βLG-isoproturon

(βLG-IP) would mimick the immunogen while βLG-chlortoluron (βLG-CLT) was chosen based on our knowledge of the cross-reactivity characteristics of isoproturon antibodies. These antibodies immobilized on a silica matrix produced an immunoaffinity cartridge efficient in extracting other compounds of the phenylurea family including chlortoluron with a recovery yield higher than 50%.[17] The number of bound herbicide derivatives per carrier protein could be determined by reacting TNBS with the remaining free lysine side chains of the conjugated protein. βLG-IP and βLG-CLT incorporated respectively 9 isoproturon and 5 chlortoluron derivatives per carrier protein. Both conjugates kept enough available amine functions for a further coupling to CNBr activated Sepharose® 4B CL.

Characteristics of Purification Gels and Set-up of the Operating Conditions

The different affinity gels were evaluated according to a procedure described in the experimental section. Non-specific retention of antibodies due to the solid support itself (cross-linked agarose) was evaluated using virgin agarose and straight βLG linked Sepharose® CL 4B (5 mg βLG/mL of gel) columns. The recovery of 99.5% and 97% respectively of an IgG solution (10 mg), passed through each blank column proved that non-specific interaction was insignificant.

The gel incorporating ligand (II) was not efficient to give specific adsorption of antibodies from a heterogeneous IgG fraction. Passing an IgG solution through this gel resulted in the retention of 95% of the percolated proteins. The washing steps with PBS buffers resulted in a recovery of than 40% of retained proteins, which indicates that the amount of adsorbed antibodies is higher than the supposed specific proportion. This result suggested that the retention of antibodies was mainly due to non-specific interactions. This fact was confirmed by a similar adsorption rate (58%) when atrazine (a non-structurally related herbicide) antibodies were passed through the column.

No concentration in isoproturon antibodies occured when comparing the percolated and the eluted fractions with KPB/ethylene glycol buffer (30% v/v) and most of the proteins were therefore non-specifically bound, probably because of the length of the hydrophobic spacer arm. It can also be explained by the structural difference between the ligand and isoproturon due to the absence of urea replaced by an amide group. This result was reproducible with several batches of gels containing the same ligand.

The gel incorporating ligand (I) showed a specific retention of isoproturon antibodies, since the effluent solution featured a lower response than the

percolated fraction when tested by a dot blot test. The bound proportion of antibodies after percolation and three washing steps with PBS buffers did not exceed 14%. None of the applied eluents resulted in the release of a peak of eluted antibodies. The different elution procedures yielded a progressive recovery reaching 98% of bound proteins. These fractions were more concentrated in specific isoproturon antibodies than an IgG fraction (comparing solutions of same protein concentration). However this gel proved to be restrictive in selecting the useful antibodies, since a significant proportion of antibodies that recognized the blotted βLG-IP conjugate was found in the effluent solution, even though the gel capacity was not exceeded.

βLG-IP and βLG-CLT gels incorporated respectively 3.6 and 0.4 μmole of herbicide derivative per mL of gel. These amounts were at least 20 times higher than the quantity of percolated IgG (25 mg/7 mL:0.166 μmole IgG / 7mL). Both sorbents allowed a specific retention of percolated antibodies as no positive dot blot response was observed with the effluent. However, none of the applied eluents recovered more than 20% of the retained IgG on the βLG-IP sorbent. It was clear that we had a system with strong affinity. Gel overloading was observed after two purification cycles. In order to ease the antibody-antigen complex dissociation, a chlortoluron derived ligand was selected assuming it would have a reduced affinity towards the anti-isoproturon antibodies. The total capacity of the βLG-CLT gel was estimated by applying various concentrations of IgG, evaluating the amount bound for each concentration and by the extrapolation of the double reciprocal plot. This capacity was estimated to be 2 mg of antibodies per mL of gel, which was 20 times less than that expected from the determination of the amount of immobilized ligands. Steric hindrance, mainly due to antibody size, may explain this difference. Several eluents were also tried with the βLG-CLT supports. Current eluents like acidic or basic buffers did not result in a significant recovery of retained antibodies. The best results were obtained with a competitive elution involving a free derivative of the antigen. The carboxylic derivative of isoproturon (IPCOOH) added at the concentration of 3.10^{-4} M to a PBS/ethylene glycol solution (70/30 v/v) afforded a recovery yield of 50%. Increasing the concentration of IPCOOH, temperature and flow rate did not result in any improvement. In spite of the 50% recovery, enough purified antibodies were obtained for a further characterization and the βLG-CLT gel could be used for up to 10 times with a satisfactory efficiency, before saturation. Attempts of gel regeneration using different agents (methanol 20% v/v/acetic acid 1% v/v); NaSCN (0.2-2M); urea 8M failed in recovering the gel capacity.

A one-step purification procedure could be developed using this βLG-CLT affinity gel, in order to isolate the specific isoproturon antibodies directly from

the crude anti-serum. The recovered amount of antibodies was twice as much with the single step procedure (5 mg of specific antibodies out of 65 mg of crude-serum proteins). This procedure was reproducible provided the gel kept its working capacity. The specific antibodies, eluted with the same agents and under similar conditions, should feature the same interactions with ligates. They would therefore provide a better reproducibility if used in analytical applications.

Characterization of the Eluted Antibodies and Improvement of the Analytical Methods

Both affinity gels (I) and βLG-CLT gave a significant enrichment in specific anti-isoproturon antibodies compared to the IgG fraction as displayed in figure 3. The ELISA titer data showed that an absorbance of 1 was obtained with 633 ng/mL IgG fraction, whereas the same response was reached with 246 ng/mL of ligand (I) refined fraction (2.6 titer increase) and only 110 ng/mL of βLG-CLT purified

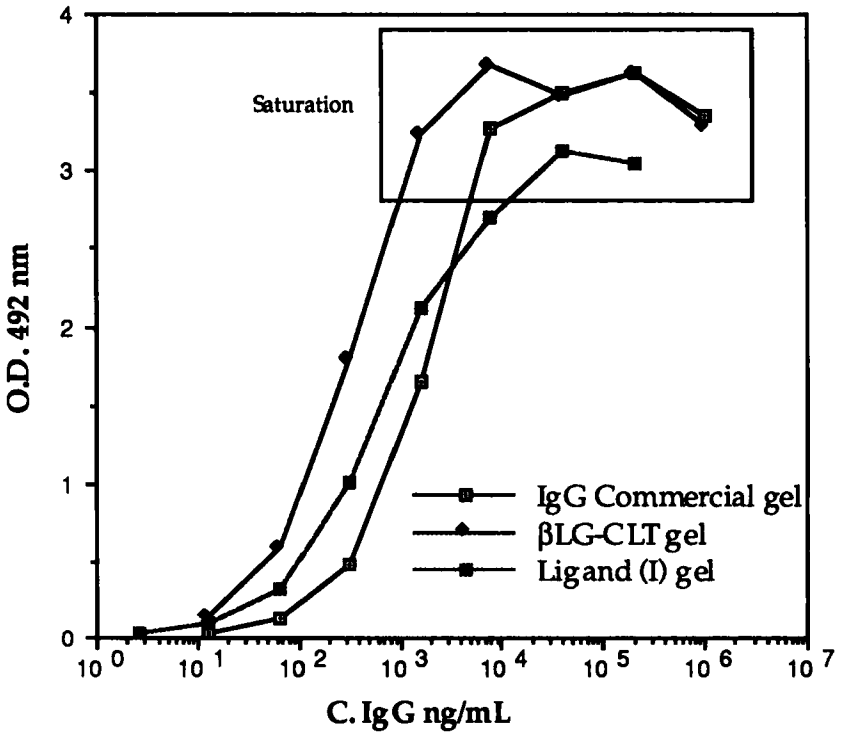


FIGURE 3 Titer determination curves of IgG and eluted fractions from both Ligand (I) and βLG-CLT gels.

antibodies: about a 6 fold titer increase. Table I displays the cross-reactivity of the eluted antibodies towards the different representatives of the phenylurea herbicide family and other non-related compounds. Cross-reactivity, evaluated in the competitive configuration with an immobilized protein conjugate of isoproturon, was found to be very weak on the whole, for all investigated compounds (<1%) with the IgG fraction. It kept the same range for both refined fractions. IC50 was increased for antibodies eluted out of gel (I) and reached 1.64 ng/mL which gives a lower selectivity of these antibodies towards the free analyte. However, it appears that IgG recovered from βLG-CLT gel feature the best characteristics with a lower IC50 for isoproturon allowing a higher selectivity and sensitivity of an enzyme immunoassay. The complete separation of these refined antibodies from other proteins in the crude serum and the efficiency of the single step purification procedure were demonstrated using comparative FPLC analyses. One peak was observed for the refined fraction, while the crude anti-serum exhibited three different groups of proteins among

TABLE I Comparison of the cross-reactivity characteristics between anti-isoproturon antibodies obtained with the different purification gels.

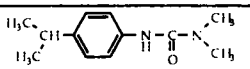
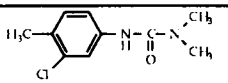
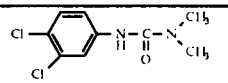
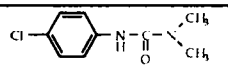
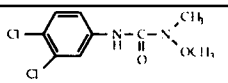
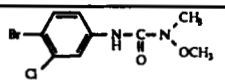
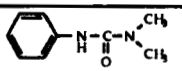
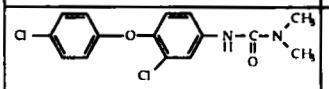
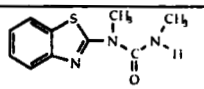
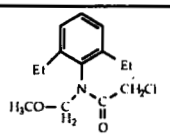
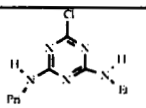
Name and structure	IgG Fraction		Gel (II) eluted fraction		βLG-CLT eluted fraction	
	IC50 (ng/mL)	%CR	IC50 (ng/mL)	%CR	IC50 (ng/mL)	%CR
 isoproturon	0.64	100	1.64	100	0.37	100
 chlortoluron	186	0.34	293	0.56	231	0.16
 diuron	208	0.31	547	0.3	205	0.18
 monuron	331	0.19	256	0.64	231	0.16
 linuron	1477	0.04	4100	0.04	1750	0.02

Table 1 (continued)

 chlorbromuron	657	0.097	n.d.	n.d.	925	0.064
 fenuron	N.R.	< 0.001	N.R.	< 0.001	N.R.	< 0.001
 chloroxuron	712	0.09	n.d.	n.d.	1233	0.03
 metabenzthiazuron	N.R.	<0.001	N.R.	<0.001	N.R.	<0.001
 alachlor	N.R.	<0.001	N.R.	<0.001	N.R.	<0.001
 atrazine	N.R.	<0.001	N.R.	<0.001	N.R.	<0.001

which only the peak corresponding to the refined fraction gave a positive dot blot test for isoproturon recognition. A significant decrease in the same peak intensity was observed for the effluent and washing solutions obtained after a purification run. These observations confirm the efficiency of the βLG-CLT gel in extracting selectively the target antibodies from the crude anti-serum.

These purified antibodies were used in both preparing immuno-affinity columns and performing the indirect enzyme immunoassay. The capacity of the immuno-affinity column is defined as the amount of pesticide trapped by 1 g of functionalized silica for 20 mg of immobilized antibodies. Two cartridges were examined for capacity determination. The first one (cartridge A; 7.5 mg protein/ 0.5 g of silica) used AvidChrom® affinity purified antibodies. The second one (cartridge B; 5.3 mg protein/0.5 g silica) used antibodies obtained after a single step βLG-CLT purification. The capacity was measured by determining the

maximum amount of isoproturon trapped by the immunosorbent. The sample volume and concentration were chosen so as to ensure that a breakthrough would occur only due to the overloading of the sorbent capacity. The capacity of cartridge-A was found equal to 3.11 ± 0.88 μg (n = 6, RSD = 28%), while that of cartridge-B with purified antibodies was equal to 8.2 ± 1.16 μg (n = 6, RSD = 14%) : a 2.6 fold increase of immunosorbent capacity. The breakthrough volume (Vb) of isoproturon (percolated volume leading to 10% breakthrough in the effluent) was determined for 2 immunoaffinity cartridges made with AvidChrom® and βLG-CLT purified antibodies (cartridge A' 3 mg antibodies/ 0.25 g silica; cartridge B' 3.2 mg antibodies/0.25 g silica). Vb was found to be equal to 125 mL for cartridge A', whereas cartridge B' allowed the percolation of 300 mL with a breakthrough less than 5% (Vb = 350 mL). Furthermore, the elution step could be standardized using 4 mL of a 80% MeOH/20% LC-grade water solution. These observations emphasize the improvement of the retention and desorption characteristics of the immunoaffinity cartridge when βLG-CLT purified antibodies are used.

The main improvement noticed when the isoproturon enzyme immunoassay was performed using the affinity purified antibodies was observed in the recovery experiments, with fortified surface water samples. The % Standard Deviation obtained for the calibration curves showed that the test is less variable with the βLG-CLT purified antibodies. A lower inter-day standard deviation was also observed. Recoveries were decreased well below 150% and ranged between 83% to 99.7%, giving a better correlation with HPLC analyses (r = 99.8%). This was particularly evident for the 2 ng/mL and the 4 ng/mL spikes. It was clear that the serum purification improved most of the assay characteristics. This conclusion is consistent with what was obtained by Assil et al for their indirect immunoassay of sulfonamides.[15]

CONCLUSION

We have synthesized different ligands for isoproturon antibody purification. Affinity ligands consisting in herbicide-protein conjugates were found to be the most efficient in developing a one step reproducible procedure. The recovered antibodies allowed a 6 fold titer increase compared to an IgG fraction obtained by a commercial gel and were evaluated for their use in both an immunoaffinity column and an enzyme immunoassay. This work shows the possible improvement of immunochemical-based environmental analytical applications if affinity purified antibodies are developed.

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