Preparation of Pentachlorophenol Derivatives and Development of a Microparticle-Based On-Site Immunoassay for the Detection of PCP in Soil Samples

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Pentachlorophenol (PCP) is used as a herbicide in agriculture and as an insecticide for termite control. Because of the apparent hazard associated with its usage, there is a need for an efficient and economic on-site screening method. A 5-min on-site test has been developed for the detection of PCP based on the OnTrak format, a successful Roche on-site test format for drugs of abuse, utilizing the principle of latex agglutination immunoassay. The test detects 1 ppm of PCP in soil samples.

Keywords: *Pentachlorophenol (PCP); on-site test; OnTrak; latex agglutination immunoassay*

INTRODUCTION

Pentachlorophenol (PCP) is used as a general herbicide in agriculture and as an insecticide for termite control in the preservation of wood. Despite the benefits of this material, excess ingestion of PCP causes lung, liver, and kidney damage (*1*). Unfortunately, high levels of PCP were found in numerous waste sites (*2*). Therefore, there is a need for an efficient and economic on-site screening method to monitor remedial actions at these sites. Immunoassays meet these requirements, and over the past 25 years they have been increasingly utilized in the field of environmental pollutant monitoring. However, the early formats of immunoassays are slower enzyme-linked immunosorbent assay (ELISA)-based methods and occasionally radioimmunoassy (RIA) (*3*). Novel formats that are much easier and quicker to perform have been developed and marketed more recently. Typically, test results can be obtained within 30 min or less with these updated formats, as opposed to the much longer time required by ELISA.

We have developed a rapid on-site test for the detection of PCP based on the OnTrak format, a successful Roche on-site immunoassay test format for drugs of abuse (*4*−*6*). Like other OnTrak immunoassays, this test is visualized by the occurrence of agglutination of PCP-derivatized microparticles, effected by the addition of the anti-PCP antibody (Scheme 1). When free PCP is present, agglutination is inhibited and the degree of inhibition correlates with the amount of free PCP present in the sample (Scheme 2), which competes for the same binding sites of the antibody as the PCP on derivatized microparticles does. The test takes only ∼5 min and has been configured to have a cutoff detection limit of 1 ppm (μg/mL) of PCP in soil samples. In this paper, we describe the design of immunogen, production of antibody, synthesis of PCP−protein conjugates, and performance of the test.

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MATERIALS AND METHODS

General. All solvents were obtained from Fisher Scientific. All flash-grade silica gel and TLC plates were purchased from E. M. Science. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), and bovine thyroglobulin (BTG) were acquired from Miles Laboratory, Calibiochem, and Sigma, respectively. 2,3,5,6-Tetrachlorohydroquinone was obtained from Sigma. Trinitrobenzenesulfonic acid (TNBS) was obtained from Pierce. Carboxyl-modified microparticles were from Serydyne. Coomassie protein assay reagent was purchased from Bio-Rad. Rabbit anti-sheep antibody conjugated with bovine alkaline phosphatase was a product of Zymed Laboratories, Inc., San Francisco, CA. Weathered soil samples consisting of 65% clay and 35% sand were purchased from Environmental Resource Associates, Arvada, CO. All other chemicals and/or reagents were from either Aldrich or Sigma. ELISA data were recorded with an SLT multiple well reader. A RaPID Assays unit and reagents were purchased from Ohmicron Environmental Diagnostics, Newtown, PA (which was merged into Strategic Diagnostics Inc., Newark, DE).

Chemical Synthesis. *(2,3,5,6-Tetrachloro-4-hydroxyphenoxy)acetic Acid (**1**).* 2,3,5,6-Tetrachlorohydroquinone (4.96 g, 20.0 mmol) was dissolved in 30 mL of a 35% aqueous NaOH solution, followed by the addition of 11.5 mL of water. The resulting solution was heated in an 80 °C oil bath. A solution of bromoacetic acid (24.1 g, 173.4 mmol) in 27 mL of water was added to the heated solution dropwise for a period of 1 h. Heavy precipitation occurred by the end of the addition, and the heating of the resulting mixture was continued at 80 °C for another 4 h. After cooling to room temperature, the crude brown material was collected by filtration, which was then purified through recrystallization in 95% EtOH solution to give 4.81 g of product (79%, based on hydroquinone): ^{1}H NMR (DMSO-d_6) δ 10.8 (s, 1 H, very broad), 4.48 (s, 2 H). FAB-MS: m/z 304.9 (M + H).

*1-[[(2,3,5,6-Tetrachloro-4-hydroxyphenoxy)acetyl]oxy]-2,5-pyrrolidinedione (**2**).* 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (1.06 g, 5.5 mmol) was added to a mixture of (2,3,5,6-tetrachloro-4-hydroxyphenoxy)acetic acid (1.53 g, 5.0 mmol) and *N*-hydroxysuccinimide (NHS) (575 mg, 5.0 mmol) in 21 mL of dry dimethylformamide (DMF). A very strong

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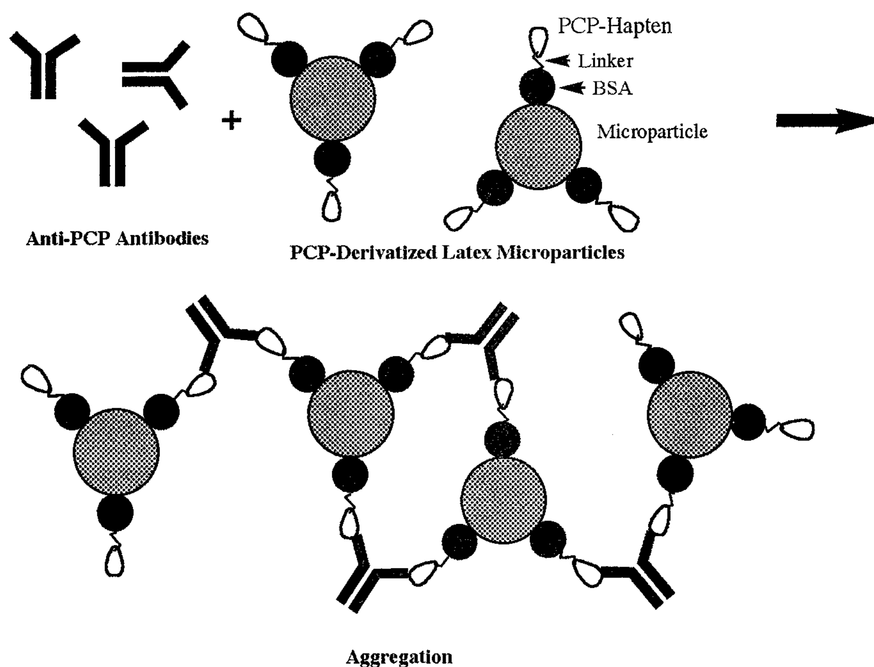
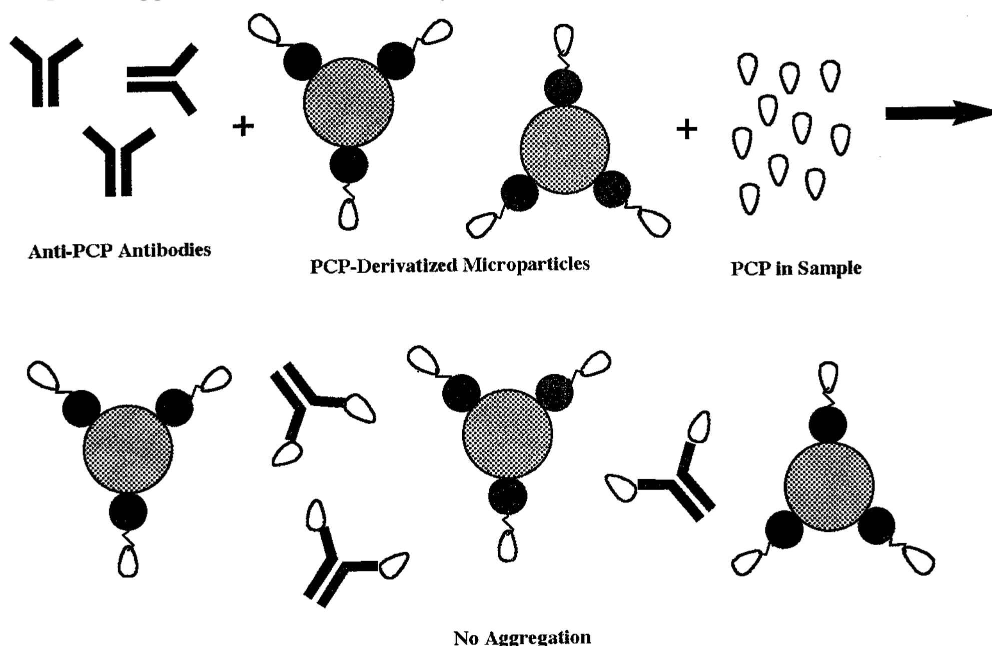
Scheme 1. Principle of Agglutination Immunoassay Negative Reaction



Scheme 2. Principle of Agglutination Immunoassay Positive Reaction



purple color appeared. After it was stirred at room temperature for 1 day, the reaction solution was evaporated in vacuo (40 °C bath). The residue was dissolved in 80 mL of methylene chloride and washed with water (three times) and brine (one time), respectively. The washed solution was dried (Na_2SO_4) and evaporated to give 1.79 g of residue. The residue was dissolved in ~4 mL of $MeOH/CH_2Cl_2$ (10:90, v/v), loaded onto a flash silica gel column, and eluted with the same solvent system. Fractions containing the product were pooled and evaporated. The residue was crystallized in a small amount of chloroform; crystals formed were collected and dried in vacuo (389 mg, 19.3%): ^{1}H NMR (acetone-d_6) δ 5.12 (s, 2 H), 2.93 (s, 4 H). FAB-MS: m/z 401.9 (M + H).

*Methyl 4-[(2,3,5,6-Tetrachloro-4-hydroxyphenoxy)acetyl]aminophenylacetate (**3**).* To a mixture of compound **1** (720 mg, 2.35 mmol), methyl 4-aminophenylacetate HCl (475 mg, 2.35 mmol), and EDC (496 mg, 2.59 mmol) was added 10 mL of DMF and 344 μL of triethylamine (2.48 mmol). The resulting solution was stirred at room temperature for 22 h and then poured into 200 mL of water. The mixture was extracted with ethyl acetate (three times). After the first extraction, the pH of the aqueous phase was adjusted to 3 using concentrated HCl. The organic extracts were combined and washed with 1 M HCl (two times), 3% $NaHCO_3$ (one time), and brine (one time). The solution was then dried and evaporated; the crystalline residue was recrystallized in $CHCl_3$/hexane (6 mL/2 mL) to give 626 mg of product (59%): ^{1}H NMR (DMSO-d_6) δ 10.07 (s, 1 H), 7.60 (d, 2 H, J = 8.4 Hz), 7.20 (d, 2 H, J = 8.4 Hz), 4.57 (s, 2 H), 3.62 (s, 2 H), 3.59 (d, 3 H).

*4-[(2,3,5,6-Tetrachloro-4-hydroxyphenoxy)acetyl]aminophenylacetic acid (**4**).* Compound **3** was dissolved in 24 mL of warm EtOH, followed by dropwise addition of 5 mL of 1 M LiOH solution. After the resulting solution was stirred at room temperature for 24 h, it was filtered and evaporated. The residue was redissolved in ~5 mL of water, and the resulting solution was acidified with concentrated HCl to pH ~1. The precipitate formed was collected and dried to yield 310 mg of gray solid (62%): ^{1}H NMR (DMSO-d_6) δ 12.32 (s, 1 H, broad),

10.99 (s, 1 H, broad), 10.06 (s, 1 H), 7.59 (d, 2 H, $J = 8.5$ Hz), 7.19 (d, 2 H, $J = 8.5$ Hz), 4.57 (s, 2 H), 3.51 (s, 2 H).

Preparation of PCP—KLH (Immunogen I). DMF (8 mL) was added to a cold KLH solution (25 mL, 10 mg/mL) in 50 mM, pH 7.5, phosphate buffer. A solution of compound **2** (101 mg) in 2 mL of DMF was added dropwise into the KLH solution, and the resulting solution was stirred at room temperature overnight. The reaction solution was transferred to a dialysis tubing with molecular weight cutoff of 2000 and dialyzed extensively against a pH 7.5, 50 mM, phosphate buffer. The dialyzed solution was filtered through a sterile 0.2 μm filter unit.

Preparation of PCP—BTG (Immunogen II). This immunogen was meant to be used as a backup and prepared using the same procedure as outlined above for the preparation of PCP−KLH immunogen, except that BTG was used in place of KLH. After dialysis, the concentration of the modified BTG was found to be 4.0 mg/mL and Lys modification 84%. The overall protein recovery was 83%.

Preparation of PCP—BSA Conjugate I. The conjugate was made using a procedure similar to the preparation of PCP−KLH immunogen, except that only 2 molar equiv of compound **2** was allowed to react with BSA in a mixture of pH 7.5, 50 mM, phosphate buffer/DMF (2.5:1, v/v).

Preparation of PCP—BSA Conjugate II. The conjugate was made using the procedure identical to the preparation of PCP−BSA conjugate I, except that compound **4** was converted to its NHS ester immediately before the conjugation with BSA and, without isolation, the NHS ester formed in DMF was directly added to a buffered BSA solution.

Characterization of the Immunogens and Conjugates. The protein concentrations were determined by using the Coomassie protein assay (*7*), and BSA was used as the reference. For immunogens, the degree of PCP hapten substitution on carrier proteins was calculated on the basis of the absorbance difference at 420 nm between the TNBS-derivatized immunogen and TNBS-derivatized native carrier protein (*8*, *9*). On the other hand, the substitution of PCP−BSA conjugate, which was used for further conjugation with microparticles, was estimated by using ELISA.

Animal Immunization. Four sheep and four goats were placed on an immunization program using a method adapted from the procedure of Erlanger (*10*). Each animal received multiple site injections across the back using 1 mg of immunogen I emulsified with complete Freund's adjuvant. At the second week, the animals received booster immunizations containing 1 mg of the immunogen emulsified in incomplete Freund's adjuvant. The boost injections were repeated twice in the following 2 weeks, followed by a monthly injection of 0.5 mg of the immunogen in incomplete Freund's adjuvant for a period of 6 months. The sheep and goats were then bled, and antisera were separated from the clot by centrifugation. Screening of the antisera was done using ELISA.

ELISA for Evaluation of Antiserum Affinity to PCP—BSA Conjugate I and the Displacement of the Latter in the Presence of Free PCP. Polystyrene microplates were coated with a conjugate I solution in phosphate-buffered saline (PBS)/0.01% azide at a concentration of 5.0 μg/mL. The PBS/azide buffer was made by adding 250 mg of KH_2PO_4, 1.38 g of Na_2HPO_4, 250 mg of KCl, 9.0 g of NaCl, and 0.01% NaN_3 into 1 L of water. The coating was conducted either at room temperature for 2 h or at 4 °C overnight. The plates were then washed three times with PBS/0.1% Tween 20, followed by the addition of 50 μL aliquots of a 1% BSA solution (for displacement evaluation, 50 μL aliquots of a 500 ng/mL solution of free PCP in 1% BSA were added instead) and 50 μL diluted solutions of an anti-PCP antiserum (in PBS containing 1% BSA and 0.01% azide), respectively. The resulting plates were incubated at 37 °C for 2 h. After five washes with PBS/Tween 20, 50 μL aliquots of a diluted solution of rabbit anti-sheep antibody coupled with bovine alkaline phosphatase were added to the wells. The plates were incubated at 37 °C for 2 h and washed three times with PBS/Tween 20, and then 50 μL aliquots of a 4-nitrophenyl phosphate solution in pH 9.8 diethanolamine buffer were added. After incubation at 37 °C for ~30 min, the enzymatic reactions were stopped by the addition of 50 μL aliquots of a 3.0 M NaOH solution and the plates were read immediately at 405 nm.

Extraction of Soil Samples. Part of the soil samples were spiked with PCP at a concentration of 4.94 μg/mL, and the value was certified with GC-MS by Environmental Resource Associates. Soil samples with 0.25, 0.50, 1.0, and 1.5 μg/mL of PCP were prepared through dilution of the certified samples with unspiked soil. All soil samples (5.0 g sample size), including an unspiked one for use as a control, were extracted, respectively, with a mixture of methanol/ethylene glycol (4:1, v/v). Aliquots (~11 μL) of the extraction solutions were directly applied onto the OnTrak plates respectively for assay; the recovery of the extraction was assessed with a RaPID Assays system for PCP manufactured by Ohmicron Environmental Diagnostics.

Development of the PCP OnTrak Immunoassay. Microparticles (0.8 μm), prepared according to a previously published procedure (*6*), were covalently coated with a set of PCP−BSA conjugate and BSA mixtures using a published procedure (*11*), respectively, where the ratios of PCP−BSA conjugate versus BSA range from 1:1, 0.5:1, 0.25:1, to 0.125:1. Each batch of the microparticles coated at a specific PCP−BSA conjugate/BSA ratio was then mixed with various dilutions (titers) of the anti-PCP antibody (antisera) and a reaction buffer (pH 7.2, 50 mM, HEPES) to effect the agglutination reaction. Each test result was visually inspected 3 min after all of the reagents were mixed. The PCP−BSA conjugate/BSA ratio and antibody titer were chosen in such a way that the assay would give the most severe agglutination (scored 4 points) in the absence of free PCP and would give no agglutination (complete inhibition, scored 0 points) once the free PCP concentration reached ≥ 1 μg/mL in the sample tested. With the assay parameters thus determined, a set of standard solutions corresponding to PCP concentrations between 0 and 1.0 μg/mL produced agglutination at various degrees, which yielded scores between 4 and 0 points.

RESULTS AND DISCUSSION

Compound **1**, a close mimic of PCP in which the para-chloro group was replaced by the oxygen, was chosen as the hapten for conjugation to carrier proteins. The compound was made from the reaction of 2,3,5,6-tetrachlorohydroquinone with a large excess of 2-bromoacetic acid using a modified procedure of the original method of Meyers et al. (*12*). The acid obtained was converted to NHS ester by using EDC as the coupling reagent; several byproducts were also produced in the reaction, resulting in a low yield of the purified NHS ester (**2**). The active ester (**2**), stable when kept desiccated and frozen, was readily utilized in the conjugation with KLH and BTG. This gave immunogens I and II, respectively, with good substitution ratios of the PCP hapten (65 and 84%, respectively) on available Lys residues of both carriers.

After extensive dialysis and subsequent filtration, the immunogen I filtrate retained a KLH concentration of 3.96 mg/mL and the overall protein recovery was 85%. The immunogen I thus prepared was given to four sheep and four goats for immunization. After 6 months, the animals were bled and the antisera were screened using ELISA. It was found that the antiserum of sheep 1877 displayed the highest titer, whereas antisera of both sheep 1877 and 1878 produced similar displacement (~50%) in the presence of free PCP molecules (Figure 1). The antiserum of sheep 1877 was therefore used in the assay as the antibody reagent.

Another key reagent of the assay is the PCP-derivatized microparticles. To prepare this reagent, the PCP

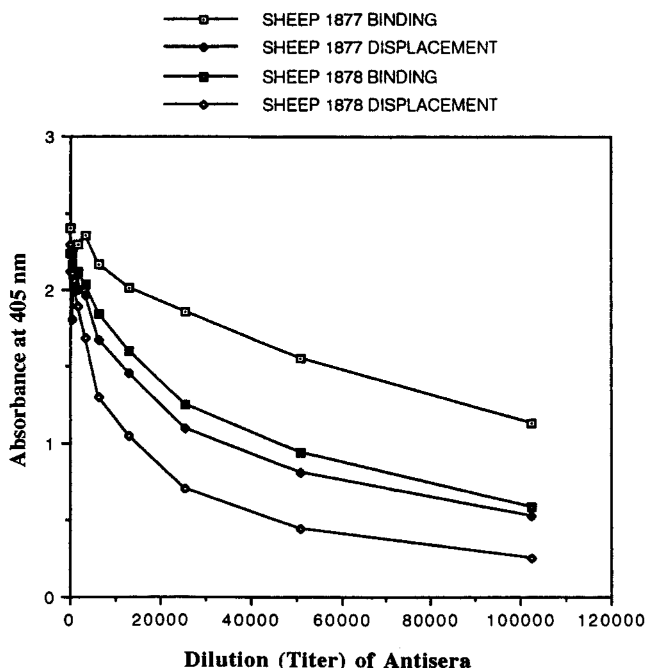
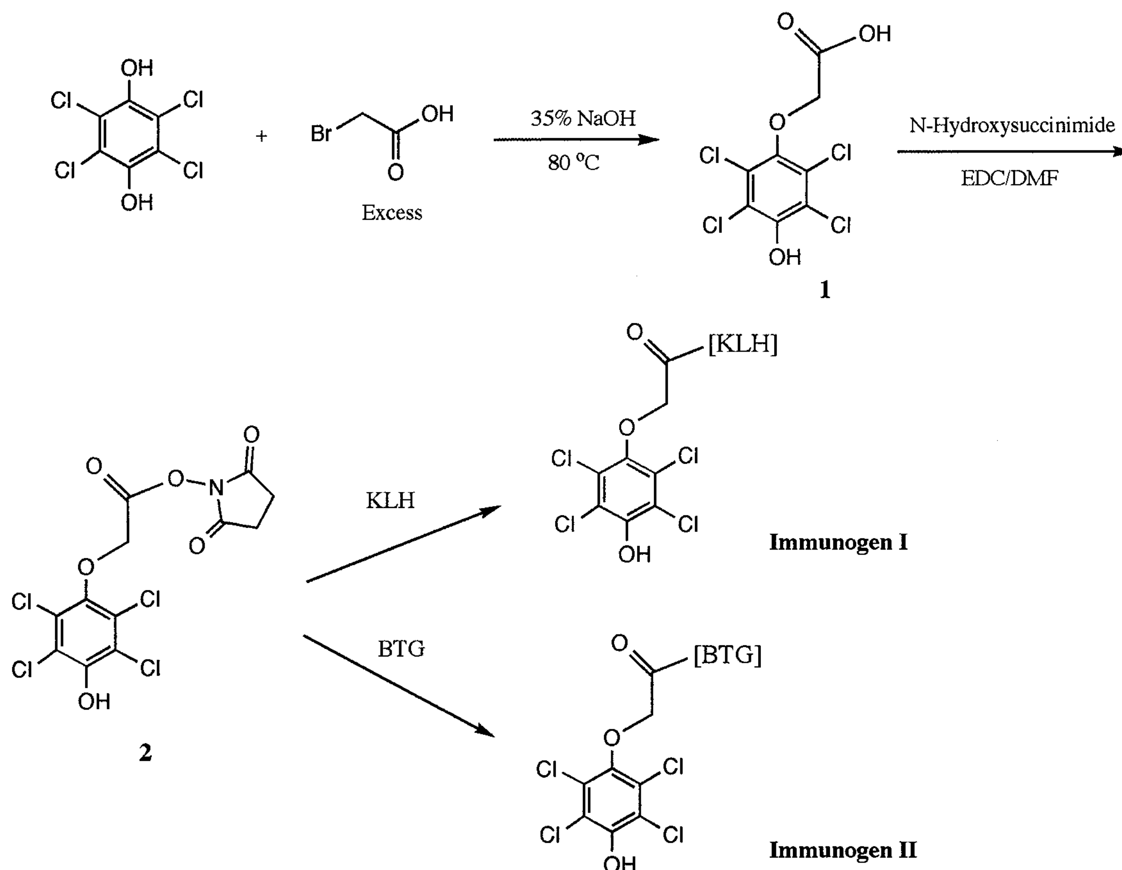


Figure 1. ELISA evaluation of antisera affinity to PCP−BSA conjugate I and displacement of the latter in the presence of free PCP (500 ng/mL).

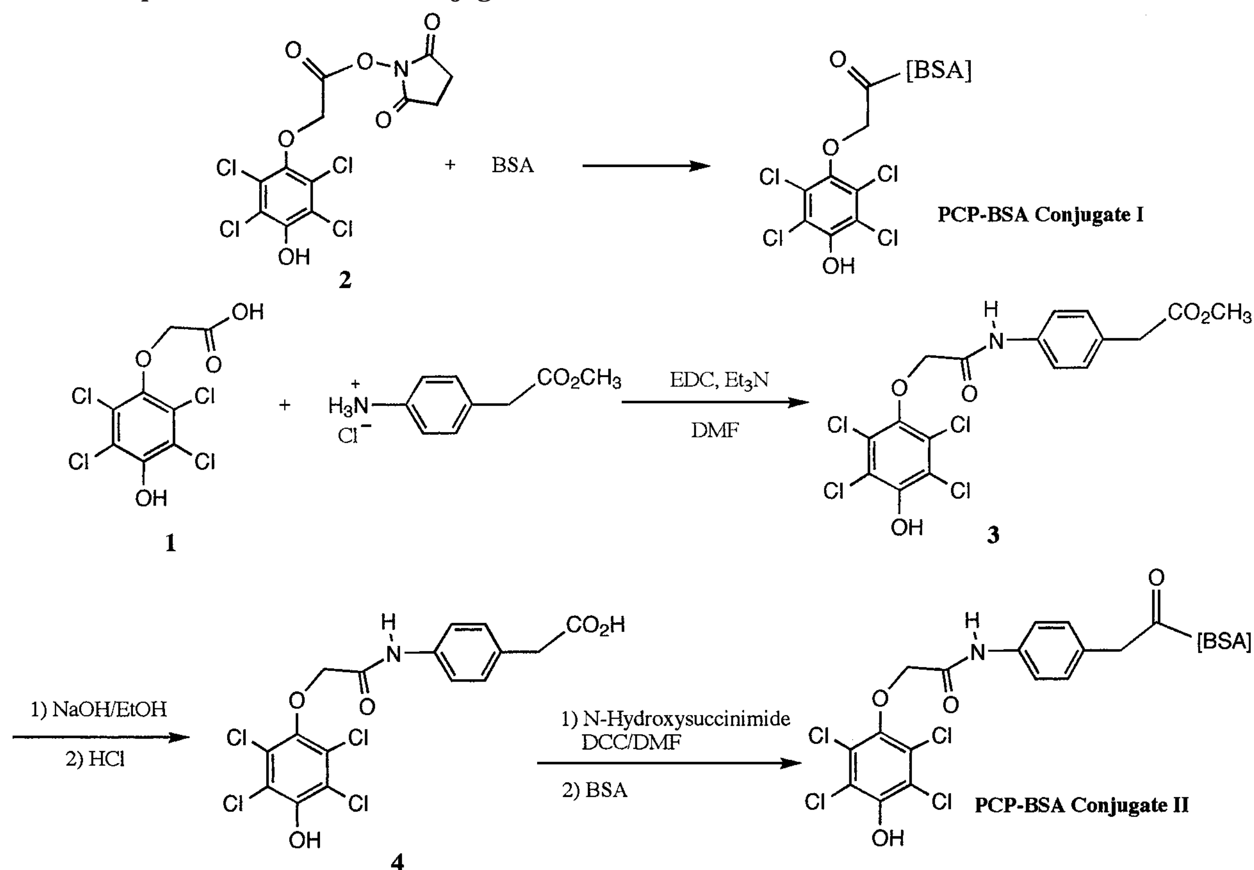
label had to be attached to a macromolecular spacer such as IgG (*11*) or BSA with a desirable molar ratio of PCP to the spacer at approximately 1:1. The PCP−spacer conjugate was then coupled to the carboxylated microparticles, as illustrated in Schemes 1 and 2. In this case, we chose BSA as the spacer and the two PCP−BSA conjugates (I and II) were evaluated in the preparation of the microparticle reagent. PCP−BSA conjugates I and II were prepared, respectively, from the conjugation of the corresponding NHS esters with BSA in a 2:1 molar ratio (Scheme 4). It was estimated by ELISA that each mole of BSA contained ~1.2 mol of PCP label in the conjugates thus formed. As opposed to the case of the two immunogens with which high PCP substitution ratios were achieved, the indirect TNBS assay is not accurate enough to measure the very low PCP substitution ratio of PCP−BSA conjugates. Both conjugates were then coupled to microparticles to produce two reagents containing different linkers. Conjugate II has an extended linker (4-aminophenylacetic acid), and it was hoped that the derivatized microparticles made from this conjugate might be more easily displaced from the antibody binding site by the free PCP molecules. The antibody used in this assay was raised against the antigen made from compound **2**, which lacks the extended linker and would therefore be expected to bind less well to conjugate II. Having such an ability to tune the degree of displacement of the derivatized microparticles is important for performance adjustment of the assay, because the degree of the displacement is directly related to the sensitivity of the assay. In the process of the assay development, it was observed, as anticipated, that the microparticle reagent derivatized with BSA−PCP conjugate II demonstrated greater sensitivity toward free PCP than did the reagent made from conjugate I. Thus, the former microparticle reagent (derivatized with conjugate II) was adopted in the OnTrak assay.

With both the antibody and microparticle reagents available, a test program with soil samples spiked with various amounts of PCP was started. The recovery of

Scheme 3. Preparation of Immunogens



Scheme 4. Preparation of PCP−BSA Conjugates



each extraction was quantitated with Ohmicron's RaPID Assays for PCP, a commercial PCP screening apparatus based on immunoassay principle, and the averaged recovery was found to be 92% with an RSD of ±10%. Aliquots of the extraction were then applied onto OnTrak assay plates, and corresponding scores were assigned according to the degree of individual agglutination on each plate. In Figure 2 are shown the results of a typical set of experiments involving the extraction and OnTrak assay of 44 soil samples. The difference between the cutoff (1.0 μg/mL) and half-cutoff (0.5 μg/mL) was readily visualized, although 2 of 10 samples spiked with 1.0 μg/mL PCP showed slight agglutination (thus, a score of 0.5 was given in these two cases). On the other hand, complete agglutination occurred when soil samples containing 1.0 ppm of atrazine (another widely utilized agrochemical), benzene, toluene, or xylenes were tested using the same procedures.

In summary, we have designed and prepared two immunogens and one of them was used in the immunization program that produced antisera with satisfactory affinity toward PCP and PCP−BSA conjugate II. The microparticle reagent was made from the coupling of PCP−BSA conjugate II to carboxylated microparticles. Agglutination of the derivatized microparticles was effected by the addition of the antibody reagent; inhibition of this process occurred when free PCP molecules were present in a sample being tested. The degree of the inhibition was proportional to the amount of free PCP, and the whole event is easily visualized within 5 min, giving rise to a fast and economic on-site PCP test.

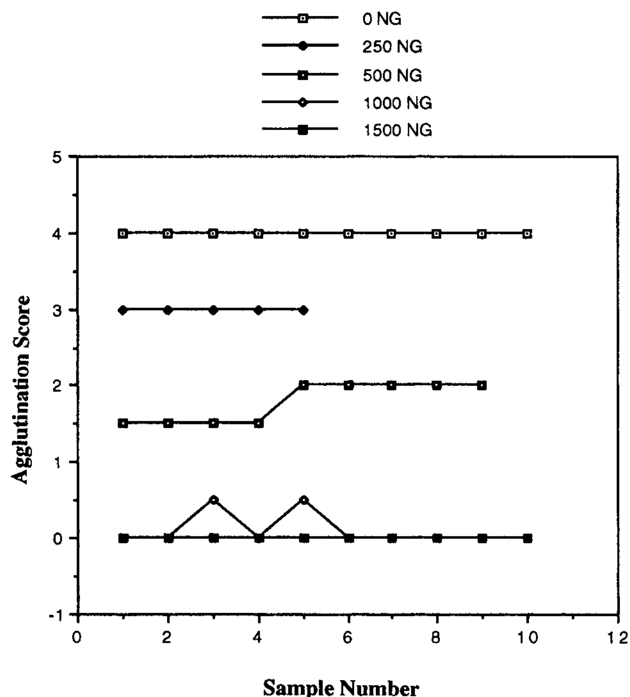


Figure 2. Agglutination scores of the PCP OnTrak assays from extracted PCP-spiked (250−1500 ng/mL) and unspiked soil samples.

ABBREVIATIONS USED

PCP, pentachlorophenol; ELISA, enzyme-linked immunosorbent assay; TLC, thin-layer chromatography; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; BTG, bovine thyroglobulin; TNBS, trini-

trobenzenesulfonic acid; PBS, phosphate-buffered saline; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; NHS, *N*-hydroxysuccinimide; DMF, dimethylformamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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