

Development of Sensitive Immunoassays for the Detection of the Glucuronide Conjugate of 3-Phenoxybenzyl Alcohol, a Putative Human Urinary Biomarker for Pyrethroid Exposure

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Pyrethroids are widely used in agriculture as insecticides. This study describes a sensitive enzyme-linked immunosorbent assay for the detection of the glucuronide conjugate of 3-phenoxybenzyl alcohol, a putative pyrethroid metabolite that may be used as a biomarker of exposure to pyrethroids. Four antisera were elicited against two different immunizing haptens. Antisera were characterized in combination with several coating haptens. The lowest IC₅₀ value (0.5 ng/mL) was obtained with antiserum 1891 and 3-phenoxybenzoic acid–BSA conjugate as the coating antigen. Antiserum 1891 was highly selective for the target compound with an overall cross-reactivity of <0.3% to structurally related compounds. The assay sensitivity was negligibly affected by pH 4–9. A 5-fold improvement in IC₅₀ was observed using a 10-fold concentrated phosphate-buffered saline as the assay buffer. Compared to assays conducted in normal phosphate-buffered saline, the maximal absorbance was almost identical. A good correlation ($r^2 = 0.99$ and 0.97 for urine samples A and B, respectively) was observed between spiked levels and the levels detected by the immunoassay.

KEYWORDS: ELISA; pyrethroid; glucuronide conjugate; human exposure; hapten

INTRODUCTION

Pyrethroids exert neurotoxic effects on the axons of the nervous system by interacting with sodium channels in insects and mammals (1). High potency in controlling a wide spectrum of insects and low toxicity to birds and mammals have made it widely accepted for application in agriculture, forestry, homes, horticulture, and public health around the world (2–4). Although pyrethroids are considered to be safe for humans, there have been concerns arising from their potential effects on human health from long-term exposure or high exposure of children. Environmental concerns include accumulation and leaching into the surface water and groundwater (5–8). Some research indicates that high exposure to pyrethroids might cause endocrine disruption, suppressive effects on the immune system, lymph node and splenic damage, and carcinogenesis (9–11). Pyrethroids are also known to be highly toxic to some aquatic species and beneficial insects. Pyrethroids have been found in various agricultural and commercial products such as vegetables, fruits, and shampoo and surface water and groundwater (12). Therefore, it is important to develop a rapid, sensitive, and efficient analytical method for both toxicological and epidemiological monitoring.

Most commercial pyrethroids are esters. These pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage to *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA) and 3-phenoxybenzyl alcohol (Figure 1). The 3-phenoxybenzyl alcohol is further processed to 3-phenoxybenzoic acid (PBA). DCCA and both the benzyl alcohol and the benzoic acid may undergo further metabolism to glucuronide, glycine, taurine, and sulfate conjugates (13–18). The cyanobenzyl alcohol from compounds such as cypermethrin and fenvalerate is expected to rearrange chemically to the corresponding benzoic acid (PBA), and thus the biomarker developed here will not be useful for type II pyrethroids. However, the glucuronide conjugate of 3-phenoxybenzyl alcohol (3-PBAc–Gluc) should be a useful biomarker for exposure to type I pyrethroids such as phenothrin and permethrin that contain a 3-phenoxybenzyl alcohol moiety.

Currently HPLC and GC-MS are the primary methods used to analyze urinary metabolites of pyrethroids (19–22). Although these methods provide accurate and reliable results, somewhat complicated sample preparation cannot be avoided. Immunoassay has proven to be a sensitive analytical method for clinical diagnostics, agriculture, environmental monitoring, and food quality assessment. Previously we reported several immunoassays for the detection of pyrethroid parent compounds (23–27), their primary metabolites, and various conjugates (28–30). Although no study has specifically determined the exact

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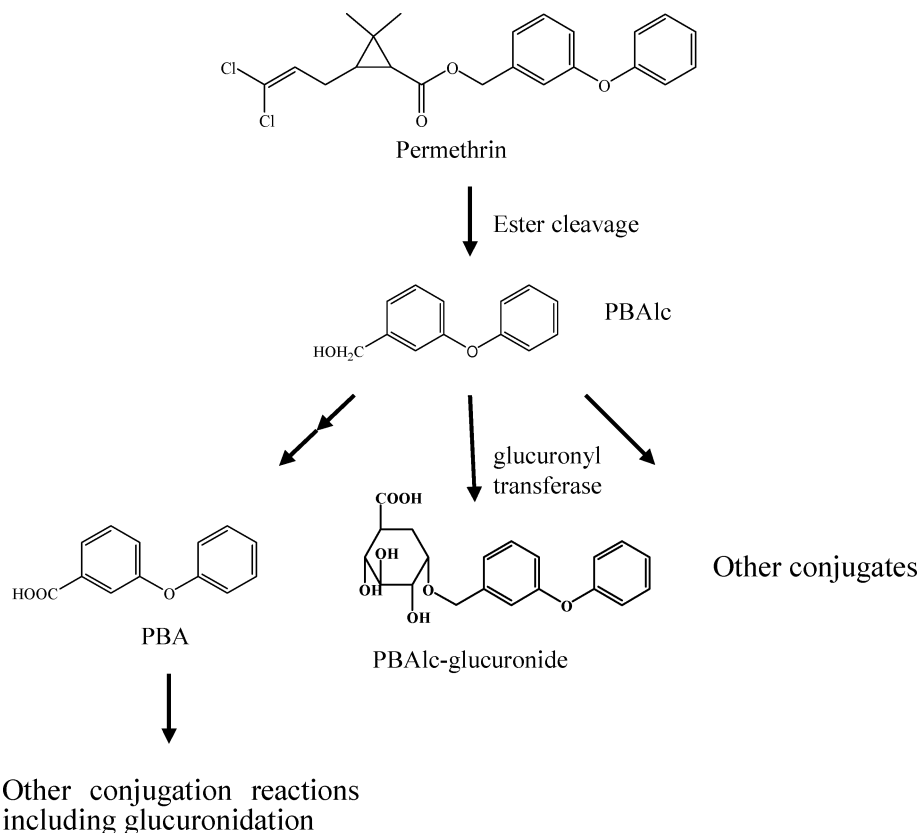


Figure 1. Mammalian metabolism of the type I pyrethroid permethrin. Conjugates vary by species, but the relative abundance of various conjugates for humans has not been determined.

conjugates of pyrethroid metabolites in humans, the development of immunoassays for all types of metabolites enhances the capability of biological monitoring for an exposure to pyrethroids because the pyrethroid metabolite profile will vary from individual to individual, and analysis for a single metabolite will not give a complete picture. In this study, we report a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the detection of the putative urinary biomarker of pyrethroid exposure, 3-PBAlc-Gluc. We describe the synthesis of the glucuronide target analyte, hapten preparation using heterobifunctional cross-linkers, antibody production against the target conjugate, assay development, and validation with urine samples.

MATERIALS AND METHODS

Chemicals. Organic chemicals for the syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany). Column chromatographic separations were carried out using Baker silica gel (40 μ m average particle size) using the indicated solvents. Heterobifunctional cross-linking reagents, (3-[2-aminoethyl]-dithio)propionic acid-HCl (AEDP) and (*N*-[ϵ -trifluoroacetylcaproyloxy]-succinic ester (TFCS), were purchased from Pierce (Rockford, IL). Other coupling reagents were purchased from Aldrich. Bovine serum albumin (BSA), thyroglobulin (Thyr), goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP), Tween 20, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO).

Instruments. ^1H nuclear magnetic resonance (NMR) spectra of compounds synthesized were obtained on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA) using tetramethylsilane as an internal standard. Electrospray mass spectra in the positive (MS-ESI $^+$) or negative (MS-ESI $^-$) mode were recorded on a Micromass

Quattro Ultima triple-quadrupole tandem mass spectrometer (Micromass, Manchester, U.K.). Melting points were determined on a Thomas-Hoover Uni-Melt apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. ELISAs were performed on 96 well microtiter plates (Nunc-Immunoplate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Menlo Park, CA) in dual wavelength mode (450–650 nm).

Synthesis of 3-Phenoxybenzyl β -D-Glucuronide (3-PBAlc-Gluc) (4; Figure 2). Methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate (**1**; 7 g, 18.6 mmol) prepared from glucuronolactone by methylation with sodium methoxide and acid-catalyzed acetylation with acetic anhydride was dissolved in dry CH_2Cl_2 (100 mL), and 45% hydrobromic acid in acetic acid (20 mL, 111.6 mmol) was added at ice temperatures. After stirring at room temperature for 3 h, the mixture was diluted with CHCl_3 and successively washed with saturated aqueous NaHCO_3 and saturated aqueous NaCl . The organic layer was filtered through activated charcoal to remove the color. After evaporation, the residue was recrystallized with ethyl ether to afford methyl (2,3,4-tri-*O*-acetyl- α -D-glucopyranosyl bromide)uronate (**2**; 5.6 g, 76%). For the Koenigs-Knorr reaction, to a solution of **2** (500 mg, 1.3 mmol) in dry CH_2Cl_2 (10 mL) were added 3-phenoxybenzyl alcohol (350 mg, 1.8 mmol), dry silver trifluoromethanesulfonate (400 mg, 1.6 mmol), and tetramethylurea (180 mg, 1.6 mmol), and the mixture was stirred under an argon atmosphere at -20°C for 12 h. The reaction was filtered and diluted with ethyl acetate (EtOAc). The organic layer was washed with saturated aqueous NaHCO_3 and applied to flash chromatography (stepwise elution with hexane/EtOAc; 20, 30, and 40% EtOAc) to afford 3-phenoxybenzyl methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate (**3**; 350 mg, 55%). Compound **3** (250 mg, 0.48 mmol) was dissolved in methanol (MeOH, 5 mL), and a 1 M sodium methoxide solution (10 mL) was added. The mixture was stirred at room temperature for 24 h, and an excess of $\text{Ba}(\text{OH})_2$ was added. The reaction mixture was stirred at room temperature for 24 h. After neutralization with Dowex-50 (H^+ -form), the mixture was filtered and evaporated in vacuo. The residue was purified with silica gel column chromatography (stepwise elution with $\text{CHCl}_3/\text{MeOH}$; 10, 20, and 30% MeOH) and with ODS column

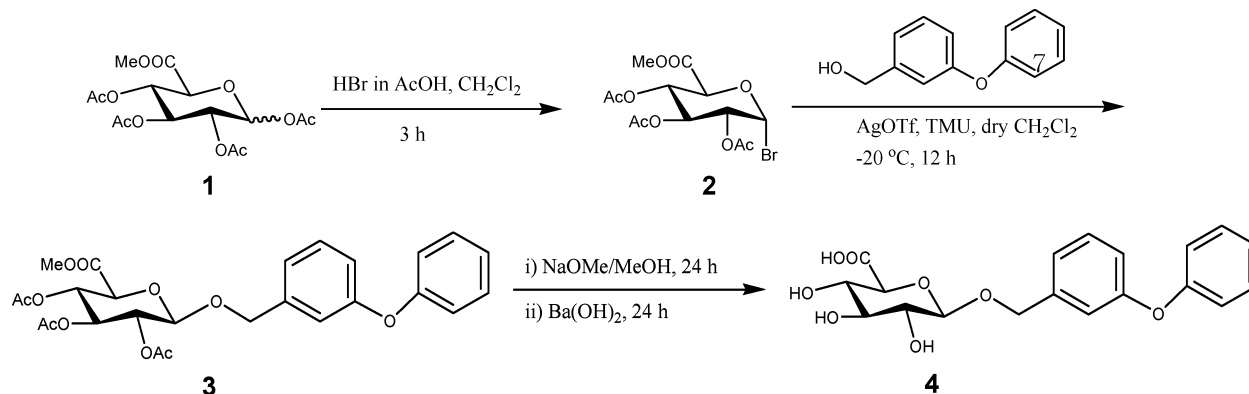


Figure 2. Scheme for synthesis of 3-PBAIc-glucuronide.

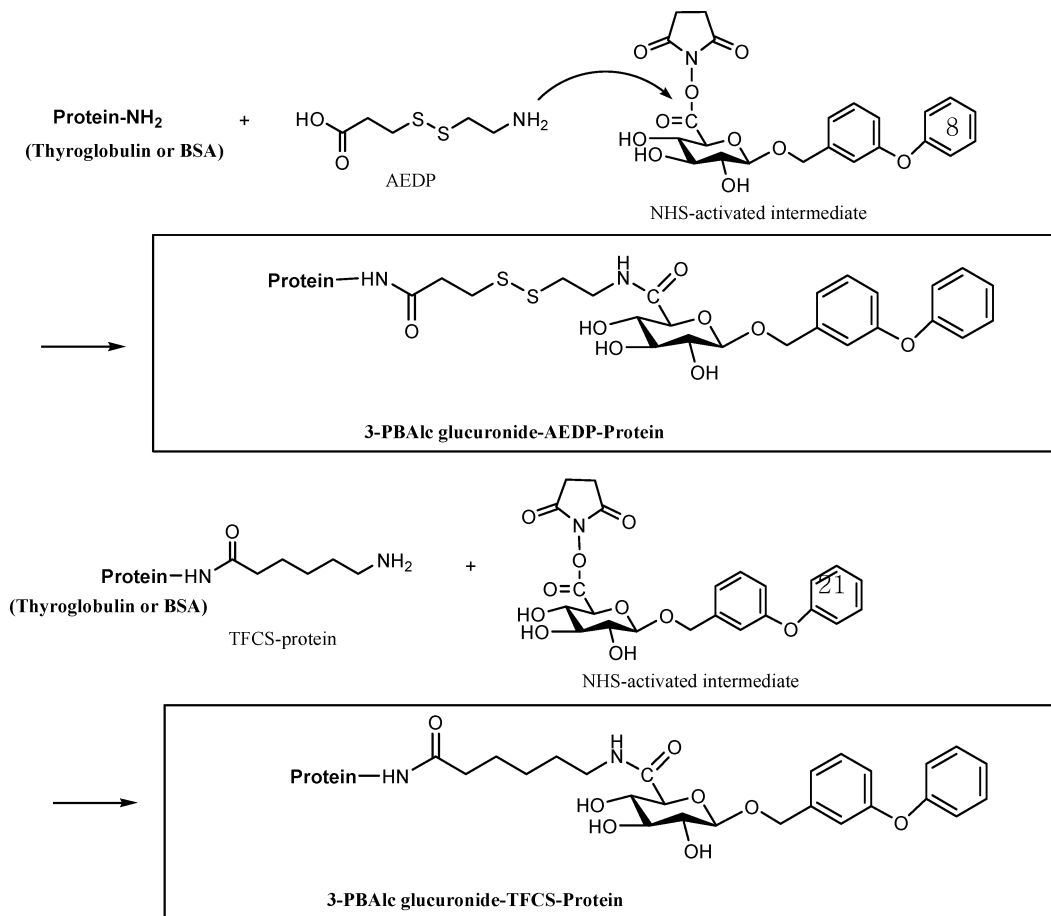


Figure 3. Conjugation route of 3-PBAIc-glucuronide to the protein (thyroglobulin or BSA) through the heterologous bifunctional linkers

chromatography (stepwise elution with acetonitrile/H₂O; 20, 50, and 80% acetonitrile). Freeze-drying gave analytically pure 3-phenoxybenzyl β -D-glucuronide (**4**) as a white powder (70 mg, 40%; total yield from **1**, 17%): ¹H NMR_H (CD₃OD), δ 3.26–3.63 (2H, Gluc H-4 and 5), 3.64 (1H, dd, $J = 7.8$ and 6.3 Hz, Gluc H-3), 4.41 (1H, dd, $J = 7.8$ and 6.3 Hz, Gluc H-2), 4.67 (1H, dd, $J = 11.7$ and 5.1 Hz, OCHH₂-Ph), 4.87 (1H, d, $J = 7.8$, Gluc H-1), 5.01 (1H, dd, $J = 11.7$ and 5.1 Hz, OCH₂HPh), 6.92–7.40 (9H, -Ph-O-Ph); HR-neg-MS, m/z [M - H]⁻ calcd for C₁₉H₁₉O₈, 375.1080; found, 375.1052.

Preparation of Immunogens and Coating Antigens. 3-PBAIc-Gluc was coupled to proteins to yield hapten-protein conjugates for immunizing and coating antigens using two heterobifunctional coupling reagents. Each heterobifunctional cross-linker possesses two different reactive groups that allow for sequential conjugations with specific functional groups of proteins. The resulting conjugates were similar except that the acid moiety of 3-PBAIc-Gluc was linked through either a -NHCH₂SS(CH₂)₂CO- or -NH(CH₂)₅CO- spacer by using AEDP or TFCS, respectively (Figure 3). 3-PBAIc-Gluc-AEDP or 3-PBAIc-

Gluc-TFCS was conjugated to Thyr for use as immunogen. For plate coating antigens, both haptens (cAg01, cAg02) and PBA (cAg05) were coupled to BSA.

Cross-Linking of 3-PBAIc-Gluc to Protein by AEDP. *N*-Hydroxysuccinimide (NHS) (0.06 mmol) and dicyclohexylcarbodiimide (DCC) (0.05 mmol) were added to 3-PBAIc-Gluc (0.04 mmol) dissolved in 0.2 mL of dry dimethylformamide (DMF). After the mixture had been stirred overnight at 4 °C under a N₂ atmosphere, the precipitated dicyclohexylurea was removed by filtration. To the activated ester solution (about 0.2 mL) were added AEDP (0.04 mmol) and triethylamine (0.04 mmol). The mixture was allowed to react at 4 °C overnight. After 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.08 mmol) in 0.5 mL DMF was added to the mixture and reacted at 4 °C overnight, the reaction mixture was added slowly to the protein solution (25 mg of protein in 5 mL of 0.05 M borate buffer at pH 8) with vigorous stirring and then allowed to stir gently at 4 °C for 24 h to complete the conjugation. This was followed by exhaustive dialysis against normal strength phosphate-buffered saline.

Cross-Linking of 3-PBAIc–Gluc to Protein by TFCS. TFCS (0.04 mmol) dissolved in 0.5 mL of DMF was added to the protein solution (25 mg in 5 mL of 100 mM sodium phosphate, 0.15 M NaCl, pH 7.2). After the mixture had been stirred at 4 °C for 2 h, the pH was adjusted to pH 7.8–8.1 with diluted 1 N NaOH and allowed to incubate for 2 h to remove the trifluoroacetyl protecting group of the cross-linker. The NHS-activated ester of 3-PBAIc–Gluc (0.04 mmol) was slowly added to the linker-attached protein solution. The reaction mixture was purified as described above.

Conjugation of PBA to Protein. NHS (0.06 mmol) and DCC (0.05 mmol) were added to PBA (0.04 mmol) dissolved in 0.2 mL of dry DMF. The activated ester of PBA was added to a BSA solution (25 mg in 5 mL of 0.05 M borate buffer at pH 8) as described above.

Hapten Density Analyses. For the determination of the amount of hapten (3-PBAIc–Gluc–linker) conjugated to protein, the BSA conjugate was dialyzed against distilled water for 24 h to remove salts, and then a powder was obtained by lyophilization. Hapten densities of the BSA conjugates were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard BSA to that of the conjugates. MALDI spectra were obtained by mixing 1 μ L of matrix (*E*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg/mL) and 1 μ L of a solution of the conjugates (5 mg/mL in 5% formic acid)

Immunization and Antiserum Preparation. Two female New Zealand white rabbits were immunized for each immunogen. Each immunogen (3-PBAIc–Gluc–AEDP–Thyr or 3-PBAIc–Gluc–TFCS–Thyr, 100 μ g) in 0.5 mL of 0.85% saline was emulsified with an equal volume of Freund's complete adjuvant, and then the emulsion was injected subcutaneously. After 3 weeks, the animals were boosted with an additional 100 μ g of immunogen that was emulsified with Freund's incomplete adjuvant (1:1, v/v). The boosts were given every 3 weeks, and blood samples were drawn 7 days after each boost to check the titers of antisera. The final antisera were collected 5 months following the first immunization. The blood was collected into a Vacutainer tube with a serum separation gel. The antisera were obtained by centrifugation and stored at –80 °C. The antiserum was used without further purification.

ELISA Buffer Solutions. Normal strength PBS (1 \times PBS; 8 g/L of NaCl, 0.2 g/L Na₂HPO₄, and 0.2 g/L of KCl, pH 7.5), PBST (PBS containing 0.05% Tween 20), carbonate–bicarbonate buffer (1.59 g/L Na₂CO₃, 2.93 g/L NaHCO₃, pH 9.6), and 0.05M citrate–acetate buffer (14.71 g/L Na₃C₆H₅O₇·2H₂O, pH 5.5) were used for immunoassay.

ELISA. Indirect competitive ELISAs were performed. The 96-well microtiter plates were coated overnight at 4 °C with 100 μ L/well of the appropriate concentration of coating antigen (cAg) in 0.1 M carbonate–bicarbonate buffer (pH 9.6). After it had been washed five times with PBST, the plate was incubated with 200 μ L/well of a 1.0% BSA solution in PBS for 1 h at room temperature. After another washing step, 100 μ L/well of antiserum diluted in PBST per well (for titration experiment) or 50 μ L/well of antiserum diluted in PBST and 50 μ L/well of standard analyte or sample solution were added and incubated for 1 h at room temperature. After the plate had been washed, 100 μ L/well of the secondary GAR–HRP (1:6000 in PBST) was added and incubated for 1 h at room temperature. The plate was washed again, and 100 μ L/well of a substrate solution [0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% of TMB in dimethyl sulfoxide (DMSO) added to 25 mL of citrate–acetate buffer, pH 5.5] was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μ L/well of 4 N H₂SO₄. The absorbance was measured using a dual-wavelength mode at 450 minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which was fit to a four-parameter logistic equation.

Assay Optimization. The assay conditions were optimized in such a way that the IC₅₀ values were minimized. This goal was achieved by screening antibodies and antigens in a two-dimensional titration for optimal dilution of cAg and antiserum. Then, competitive inhibition curves were obtained for different antiserum and cAg combinations, and the combination with the lowest IC₅₀ was selected for further assay development.

Cross-Reactivity (CR). The optimized assay was assessed for cross-reactivity by using standard solutions of the analytes and other

structurally related compounds (listed in **Table 3**). The CR was obtained by comparing the IC₅₀ values of the 3-PBAIc–Gluc standard to the tested compounds, where %CR = (IC₅₀ of 3-PBAIc–Gluc/IC₅₀ of tested compound) \times 100.

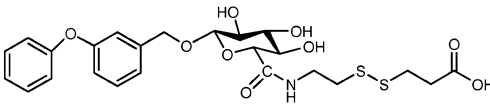
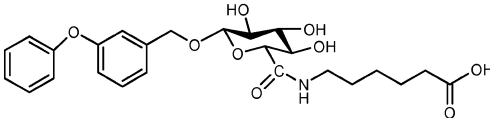
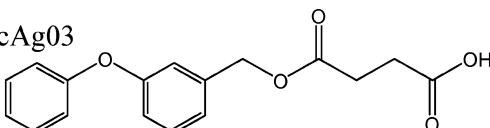
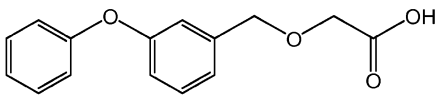
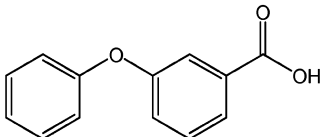
RESULTS AND DISCUSSION

Synthesis of Target Analyte and Hapten. It is known that the main detoxification reaction of carboxylic acid containing xenobiotics is conjugation either with an amino acid to form a peptide or with glucuronic acid to form a glucoside. In this respect, although the predominant type of conjugation for metabolites of pyrethroids has not been elucidated, 3-phenoxybenzoic acid is known as a main metabolite of pyrethroids and has been found in the general population at levels around 3 ppb (31). This metabolite arises from α -cyanopyrethroids by rearrangement of the corresponding cyanohydrin. It also can arise from PBAIc-containing pyrethroids by a two-step oxidation of PBAIc to PBA (**Figure 1**). PBA can be conjugated as a glucuronide, but acid glucuronides are less stable than alcohol glucuronides and also tend to transesterify around the glucuronide ring. We targeted the glucuronide of PBAIc as a possible biomarker which could distinguish among pyrethroids that hydrolyze primarily to PBAIc, PBA (ones with α -cyano moieties), or other alcohol moieties. Alcohol glucuronides are generally stable conjugates in the absence of glucuronidases. Thus, the development of a specific assay for each possible conjugate may be ideal for exposure monitoring.

The primary goals of this study were to synthesize the glucuronide target compound and develop an immunoassay specific to the 3-PBAIc–Gluc conjugate. The target compound was first enzymatically synthesized with uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) and 3-PBAIc as the substrate for glycosyl-*S*-transferase in mouse microsomes. Because the yield was low from the enzymatic synthesis, a chemical synthetic approach was taken. The chemical synthesis was based on the methods of Bulgianesi and Shen (32) and Sone and Misaki (33). The target analyte, 3-phenoxybenzyl β -D-glucuronide, was synthesized as shown in **Figure 2**. The Koenigs–Knorr reaction was used in the glucuronidation between the O-protected methyl D-bromoglucofuranate and 3-phenoxybenzyl alcohol via its silver salt. For the Koenigs–Knorr reaction, 1-bromo derivatives of the glucuronic ester are necessary as common intermediates.

To generate a specific antiserum for an analyte, the site for the linker attachment must be selected to maintain the unique target structure as distal as possible from the linker site while minimizing structural conformation changes from the modification. The phenyl ring is a potential site for linker attachment. However, attachment at this site may result in antibodies with high CR to other urinary phenolic glucuronides. Because the phenoxybenzyl ring is the unique structural feature of the main metabolite and because the high polarity of the glucuronide may elicit a strong interaction for antibody recognition, the carboxylic acid on the glucuronide was finally selected for the linker introduction. Direct conjugation of the carrier proteins to the carboxylic acid may not allow the glucuronide to be easily accessible for antibody recognition, possibly due to steric hindrance caused by bulky carrier proteins. This hypothesis is supported by our observation that the antisera against *p*-aminophenyl- β -D-glucuronide–Thyr without a linker space between the hapten and carrier protein had very high IC₅₀ values (240–400 ng/mL) (34). Therefore, we used two types of commercial cross-linkers, AEDP and TFCS, to provide space between the hapten and carrier proteins (**Figure 3**). These cross-linkers

Table 1. Titration Summary for Anti-3-PBAIc–Gluc Antisera

Coating Antigens	Antisera			
	1890	1891	1892	1893
<p>cAg01</p> 	++++	++++	+	+
<p>cAg02</p> 	++++	++++	++++	++++
<p>cAg03</p> 	+++	+++	-	-
<p>cAg04</p> 	+++	+++	-	-
<p>cAg05</p> 	++	++	-	-

^aThe data shown are at a cAg concentration of 1 μ g/mL and an antiserum dilution of 1:20000; –, absorbance < 0.2; +, absorbance = 0.2–0.5; ++, absorbance = 0.5–0.7; +++, absorbance = 0.7–1.0; +++++, absorbance > 1.0. Antisera 1890 and 1891 are derived from 3-PBAIc–Gluc–AEDP–Thyr and antisera 1892 and 1893 from 3-PBAIc–Gluc–TFCS–Thyr. Coating antigens are derived from conjugation of the hapten shown to BSA.

contain a primary amine functional group that reacts with the carboxylic acid in the target molecule and a carboxylic acid group that reacts with the lysine residues in the protein. We used an active ester method to generate the amide linkage between the hapten and the protein because of the precise control and high yield of the reaction and to avoid raising antibodies to protein–urea complexes, which sometimes result from the use of water-soluble carbodiimides.

The MALDI-TOF-MS results showed the successful conjugation of 3-PBAIc–Gluc to BSA with the two linkers resulting in molar ratios of hapten to BSA of 23:1 and 15:1 for 3-PBAIc–Gluc–AEDP and 3-PBAIc–Gluc–TFCS, respectively.

Titers and Screening of the Antisera. Four antisera were obtained against two immunizing haptens (sera 1890 and 1891 from 3-PBAIc–Gluc–AEDP and sera 1892 and 1893 from 3-PBAIc–Gluc–TFCS). A checkerboard titration method was

used to screen different combinations of those antisera with the various coating antigens (cAgs). As shown in **Table 1**, homologous combinations (cAg01 and cAg02 with antisera) showed higher titers than heterologous combinations (cAg03, cAg04, and cAg05 with antisera) due to the structural homology of cAgs with the immunizing antigens. All antisera showed high titers for cAg02, whereas antisera 1892 and 1893 showed very low binding recognition for cAg01. For other tested cAgs with the phenoxy moiety, antisera 1890 and 1891 showed measurable recognition. However, the other two sera had negligible binding. This result indicates that antisera appear to mainly bind the phenoxy moiety of the compounds. Thus, antisera 1890 and 1891 were selected for further screening. Competitive binding of antisera 1890 and 1891 against the free target analyte was evaluated with each individual cAg. Although there was no statistically significant difference in IC_{50} values between sera

Table 2. Selected Competitive ELISA Results

antiserum/cAg	ABS _{max} (A)	slope (B)	IC ₅₀ (C) ^a (ng/mL)	ABS _{min} (D)
1891/cAg01	0.5	0.7	5.0	0.08
1891/cAg02	0.6	0.6	9.5	0.07
1891/cAg03	0.8	0.8	2.0	0.3
1891/cAg04	0.8	0.6	1.7	0.2
1891/cAg05	0.5	0.8	1.3	0.04
1890/cAg01	0.4	0.7	25.6	0.1
1890/cAg02	0.4	0.7	24.6	0.08
1890/cAg03	0.6	0.8	2.0	0.4
1890/cAg04	0.6	0.6	4.4	0.3
1890cAg05	0.8	0.6	6.2	0.01

^a Each value represents the mean value of four replicates.

Table 3. Summary of CR of 3-PBAIc-Gluc Assay

chemical	IC ₅₀ (ng/mL)	CR (%)
3-PBAIc-glucuronide	1.5	100
cypermethrin	>2500	<0.06
permethrin	>2500	<0.06
esfenvalerate	>2500	<0.06
cyfluthrin	>2500	<0.06
<i>trans</i> -DCCA	>2500	<0.06
<i>cis</i> -DCCA	>2500	<0.06
<i>trans</i> -DCCA glycine	>2500	<0.06
<i>p</i> -nitrophenyl glucuronide	>2500	<0.06
PBA	934	0.16
4-hydroxybenzoic acid	>2500	<0.06
PBA-glycine	705	0.21

1890 and 1891 for all cAgs, in general, the trend showed that heterologous assays had relatively lower IC₅₀ values than homologous systems. More sensitive assays were obtained with antiserum 1891 than with antiserum 1890. Among the five tested cAgs, the most sensitive assay was obtained using cAg05 and antiserum 1891 (IC₅₀ of 1.3 ng/mL). For both antisera, cAg03 and cAg04 showed good sensitivity but also a high background signal. Thus, cAg05 was finally selected for coating (Table 2).

CR. CR was evaluated with four parent pyrethroids (cypermethrin, permethrin, esfenvalerate, and cyfluthrin), their metabolites (*trans*-DCCA, *cis*-DCCA, *trans*-DCCA-glycine, PBA, and PBA-glycine), and structurally related compounds (*p*-nitrophenyl glucuronide and 4-hydroxybenzoic acid). Antiserum 1891 is highly selective for the target compound with negligible recognition of the other tested compounds (CR < 0.3%). Although the phenoxybenzyl group appears to be necessary for antibody recognition, an extremely low CR for PBA and *p*-nitrophenyl glucuronide indicates that the phenoxybenzyl and glucuronide are both required for a high binding of antiserum 1891 (Table 3).

Matrix Effects. High tolerance of an assay to the changes in pH and ionic strength is desirable because this assay is developed for direct detection of the metabolite in urine. Assay performance under various pH and ionic strength conditions was determined (Figure 4A). pH had little effect on the assay sensitivity. Compared to pH 7.0, an approximately 10% suppression of antibody binding was observed at pH 4, 5, and 6. No changes in maximal absorbance were observed at pH 8 and 9. Although a 2-fold lower IC₅₀ value was observed at pH 7.0, this was not statistically significantly different. This indicates that changes of pH in the tested range would not affect the accuracy for the quantitation of the target compound. The assay was highly tolerant to changes in ionic strength (Figure 4B). In fact, as the ionic strength increased the IC₅₀ decreased compared to the IC₅₀ (2.5 ng/mL) at 1× PBS. Sensitivity was

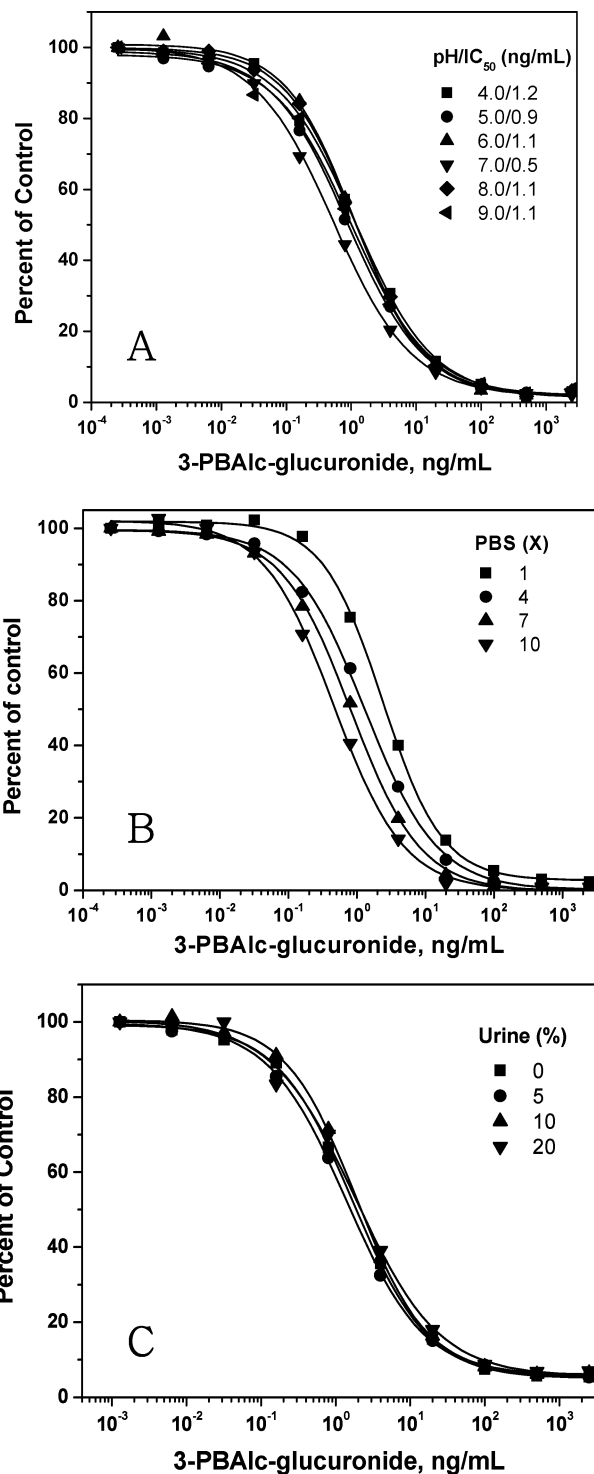


Figure 4. ELISA competition curves of 3-PBAIc-Gluc prepared at various (A) pH values, (B) ionic strengths, and (C) percentages of urine. Reagent concentrations were as follows: cAg, 0.25 μ g/well of PBA-BSA; 1:4000 final dilution, Ab 1891; and GAR-HRP, 1:6000.

5-fold lower at 10× PBS. The antibody maintained 85% binding capability so that thereafter assays were performed with 10× PBS containing 0.05% Tween 20. This effect of ionic strength could be due to a better buffering capacity of 10× PBS.

The effect of urine as a matrix was evaluated (Figure 4C). In tests with four different urine samples, the assay parameters were unchanged at 5% urine, but the antibody binding was slightly suppressed at dilutions of 10 and 20%. Nevertheless, the IC₅₀ values were almost identical. Thus, to use urine samples

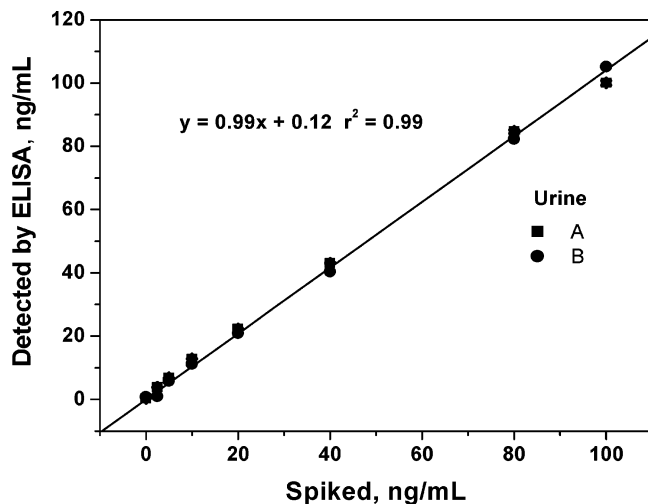


Figure 5. Relationship between analyte levels spiked into urine and measured by ELISA. Two urine samples from the two persons without known exposure to pyrethroids were used and are indicated in solid squares and solid circles.

at a 10 or 20% dilution, use of a control urine sample for the construction of standard curves may enable us to accurately detect analyte without further sample preparation or concentration.

Although a 5-fold lower IC_{50} value was observed with $10\times$ PBS, this does not indicate that the assay with $10\times$ buffer is more tolerant to the urine matrix. We compared IC_{50} values with $1\times$ and $10\times$ PBS buffer containing different percentages of urine (data not shown). Very similar matrix effects were observed, but the assay with $10\times$ PBS buffer showed consistently lower IC_{50} values for each urine percent. Thus, the assays were carried out with $10\times$ PBS. The competition curve obtained with $10\times$ PBS buffer for the target compound showed that the calibration range is approximately 0.1–5.0 ng/mL with IC_{50} of 0.5 ng/mL.

Assay Validation. The assay validation was performed in a blind fashion by direct dilution of urine samples, which were spiked with PBA1c–Gluc concentrations ranging from 0 to 100 ng/mL (Figure 5). The linear regression analysis of ELISA results showed a good correlation ($r^2 = 0.99$) between spiked and detected levels. All recoveries were $>86\%$ of the spiked values. These results demonstrated that this assay is able to accurately detect pyrethroid metabolites at trace levels in urine samples.

Conclusion. A sensitive and selective immunoassay for a putative urinary metabolite of some type I pyrethroids, 3-PBA1c–Gluc has been developed. Heterologous ELISA with the use of cAg05 as a coating antigen and serum 1891 resulted in the lowest IC_{50} of 2.5 ng/mL. The assay run in $10\times$ PBS showed 5-fold lower IC_{50} values than that with $1\times$ PBS. The assay was remarkably robust, tolerating pH values between 4 and 7 and ionic strengths up to $10\times$ PBS. The assay could tolerate up to 5% urine concentration. The maximal signals were slightly suppressed at 10 and 20% urine concentrations. However, IC_{50} values remained almost identical. The optimized assay had an IC_{50} value of 0.5 ± 0.03 ng/mL with a lower detection limit of 0.1 ng/mL. This ELISA was successfully applied to quantitate low parts per billion of analyte in spiked urine.

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