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A Sensitive Class Specific Immunoassay for the Detection of Pyrethroid Metabolites in Human Urine

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The pyrethroids are one of the most heavily used insecticide classes in the world. It is important to develop sensitive and rapid analytical techniques for environmental monitoring and assessment of human exposure to these compounds. Because major pyrethroids contain a phenoxybenzyl group and phenoxybenzoic acid (PBA) is a common metabolite form or intermediate, PBA might be used as a biomarker of human exposure to pyrethroids. A sensitive and selective immunoassay for the common pyrethroid metabolite PBA was developed. Rabbits were immunized with 3-[4-(3-carboxyphenoxy)phenoxy] N-thyroglobulin ethylamine. All sera were screened against numerous coating antigens. The assay with the least interference and the best sensitivity was optimized and characterized. The average IC_{50} for free PBA was 1.65 ng/mL. No cross-reactivity was measured to parent pyrethroids and other metabolites. Urine matrix effects can be eliminated by simple dilution. Results from urine samples from exposed workers suggest that this PBA immunoassay might be suitable as a monitoring tool for human exposure to pyrethroids.

Introduction

Pyrethroids have been widely used in agriculture, forestry, horticulture, animal and public health, and homes all around the world (*1*). They are likely to become more widely used as organophosphate insecticides are phased out due to the concerns regarding their safety. A variety of personnel are exposed to pyrethroids during manufacture and application, diet, and drinking water. Although these compounds are widely considered safe for humans (*2*), numerous studies have shown that very high exposure to pyrethroids might cause potential problems to man. Such effects include suppressive effects on the immune system (*3*, *4*), endocrine disruption (*5*), lymph node and splenic damage, and carcinogenesis (*6*). Therefore, it is important to develop a rapid, sensitive, and efficient analytical method for both toxicological and epidemiological monitoring.

A generalized pathway of pyrethroid metabolism in mammals is shown in Figure 1. Pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage, followed by various species-dependent conjugations such as to glucuronide, glycine, taurine, and sulfate (*7–10*). Current analytical methods for pyrethroid metabolites in urine rely upon multistep sample cleanup procedures including hydrolysis, extraction, and derivatization. Samples are then analyzed using either HPLC (*11*) or GC-MS with a detection limit of 0.5 μg/L urine (*12*, *13*). These methods are expensive and relatively time-consuming. Immunoassay is a leading method for rapid assessment of exposure to agrochemicals by detecting key urinary biomarkers of exposure.
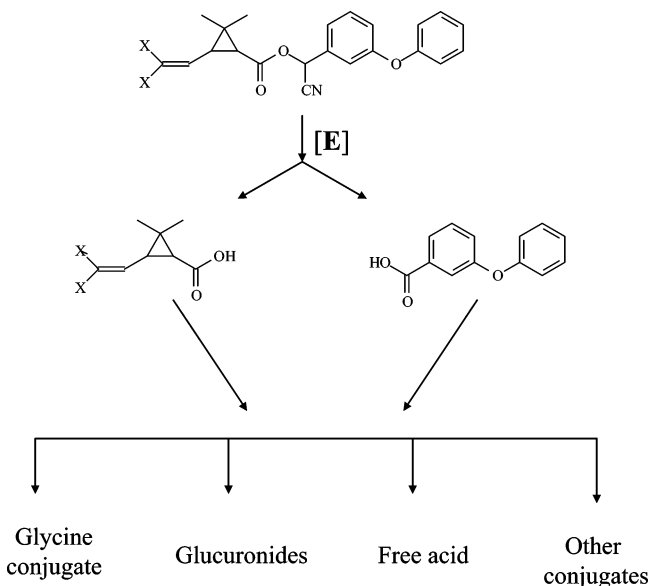

Figure 1. Pyrethroid metabolism pathway in mammals.

Because major pyrethroids such as permethrin, cypermethrin, and cyfluthrin contain the PB[1] group and PBA is a common metabolite form or intermediate, PBA conjugates might be suitable biomarkers of human
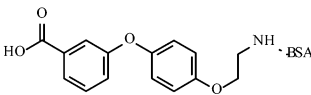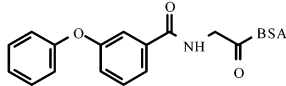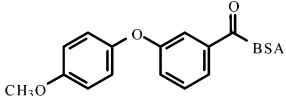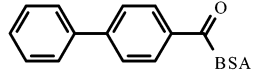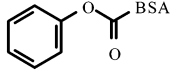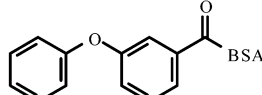
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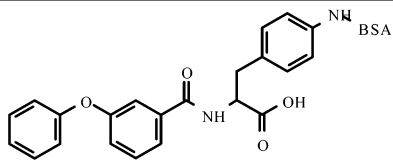
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[1] Abbreviations: Ab, antibody; BSA, bovine serum albumin; cAg, coating antigen; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; FAB-HRMS, fast atom bombardment high-resolution mass spectrum; FPBA, 4-fluoro-3-phenoxybenzoic acid; FPBAG, *N*-(4-fluoro-3-phenoxybenzoyl)glycine; GAR-HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; IC_{50}, the concentration of analyte giving 50% inhibition; LOD, limit of detection; LOQ, limit of quantitation; NHS, *N*-hydroxysuccinimide; PB, phenoxybenzyl; PBA, phenoxybenzoic acid; PBAG, *N*-(3-phenoxybenzoyl)glycine; PBST, phosphate-buffered saline with 0.05% of Tween 20; RT, room temperature; TMB, tetramethylbenzidine.

Table 1. Titration Summary for anti-PBA Antisera[a]

Coating Antigens	Structures	Ab 294	Ab 295
cAg **01** Compound **6**-BSA		++++	+++
cAg **02** PBA-glycine-BSA		+++	+
cAg **03** 4-methoxy-PBA-BSA		+	-
cAg **04** 4-biphenyl benzoic acid-BSA		-	-
cAg **05** phenoxyacetic acid-BSA		-	-
cAg **06** 3-PBA-BSA		+++	+
cAg **07** *N*-(3-Phenoxybenzoyl)-4-amino-*L*-phenylalanine-BSA		+++	+

[a] The data shown are at a cAg concentration of 0.1 μg/mL and an Ab dilution of 1:10 000; −, absorbance < 0.25; +, absorbance 0.25−0.50; ++, absorbance 0.50−0.75; +++, absorbance 0.75−1.00; and ++++, absorbance > 1.00.

exposure. In the previous study, we developed a sensitive and selective immunoassay for detecting one of the possible pyrethroid metabolites, a PBA−glycine conjugate (*14*). However, no study has specifically determined the nature of the conjugates of pyrethroid metabolites in humans. More likely, the pyrethroid metabolite profile will vary from individual to individual. An assay detecting a single conjugate such as PBA−glycine may not be sufficient for exposure monitoring. A sensitive and specific PBA assay will be more significant since it allows us to detect all conjugation forms after a hydrolysis treatment, and it will complement conjugate specific assays. A PBA assay could be used as a generic biomarker of human exposure to pyrethroids. In this study, an immunoassay to PBA was developed and characterized. Urine matrix was evaluated, and the resulting assay was applied to urine samples from exposed workers.

Experimental Procedures

Chemicals. The standards cypermethrin, fluvalinate, fenvalerate, and permethrin were obtained from Riedel de Haen (Seelze, Germany). Esfenvalerate was synthesized as described by Shan et al. (*15*) with a purity of >99% based on analytical data. BSA, thyroglobulin (Thyr), Tween-20, 3,3′,5,5′-TMB, and GAR-HRP were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), and Lancaster Synthesis, Inc. (Windham, NH). Preparation of cAgs cAg **02**, cAg **06**, and cAg **07** was reported earlier (*14*) (Table 1). FPBA was prepared in this laboratory with a purity of 96.4%. Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel, packed in glass columns of such diameter to give a column

height/diameter ratio of ∼7. The → notation denotes a stepwise solvent gradient.

Instruments. Proton NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from the internal standard, tetramethylsilane. TLC utilized 0.2 mm precoated silica gel 60 F254 on glass (E. Merck, Darmstadt, Germany), and detection was made by UV light or iodine vapor stain. Fast atom bombardment high-resolution mass spectra (FAB-MS) were obtained on a ZAB-2SE mass spectrometer (VG Analytical, Wythenshawe, U.K.), using high energy cesium ions at a density flux of 1−2 mA and 35−38 kV to generate secondary [MH]$^+$ ions. The liquid matrix was glycerol or 3-nitrobenzyl alcohol, and cesium iodide was used for mass calibration at a dynamic resolution of 5000:1. ELISAs were carried out with 96 well microtiter plates and read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Synthesis and Verification. Syntheses of hapten **6** and FPBA−glycine were carried out as outlined in Figures 2 and 3. All reactions were straightforward using well-known procedures, and yields were good. NMR spectral data supported all structures, and mass spectra further supported the structure of **6**.

1. Synthesis of Hapten 6. 1.1. 3-(4-Methoxyphenoxy)-benzoic Acid (1). 3-(4-Methoxyphenoxy)benzaldehyde (5.0 g) was mixed with 50 mL of distilled water containing 1.0 g of K_2CO$_3$. At RT, a catalytic amount of tetrabutylammonium chloride was added, and then, 1.0 g of KMnO$_4$ was added in portions with vigorous stirring. The reaction mixture turned to a purple color and was stirred for another 60 min. A small amount of solid NaHSO$_3$ was added and stirred for 15 min to remove unreacted KMnO$_4$. The mixture was filtered through Celite, and the filtrate was acidified with 1 N HCl to precipitate the acid. Then, the precipitate was filtered and dried to give 4.02 g of
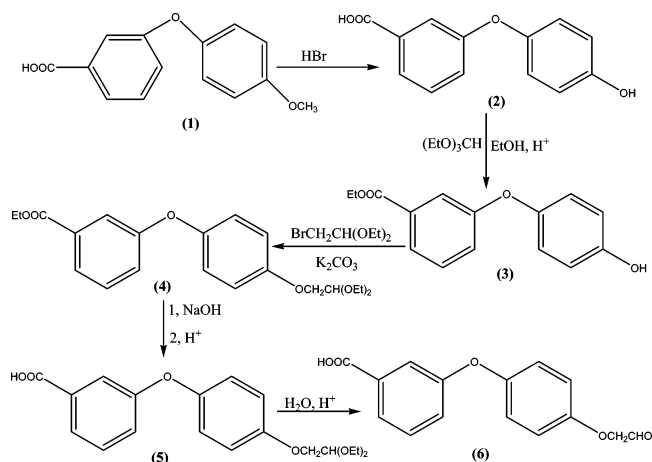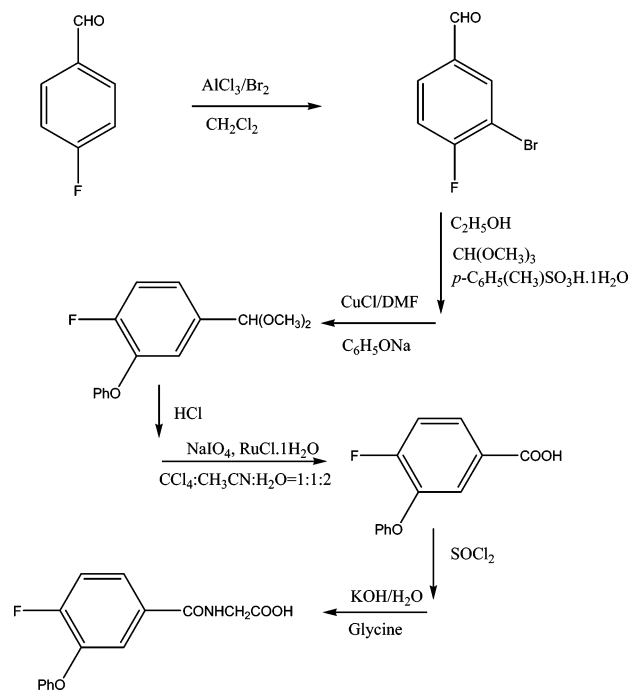
Figure 2. Scheme for synthesis of hapten 6.

Figure 3. Scheme for synthesis of FPBA-glycine.

compound **1**. TLC R_f 0.49 (ethyl acetate:hexane = 1:1, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 3.77 (S, 3H, CH$_3$O), 6.98–7.08 (m, 4 H, Ar), 7.21–7.65 (m, 4 H, Ar), 13.1 (bs, 1 H, COOH).

1.2. 3-(4-Hydroxyphenoxy)benzoic Acid (2). A mixture of **1** (3.04 g, 12.4 mmol), concentrated hydrobromic acid (30 mL), and xylenes (1.5 mL) was refluxed for 12 h. The mixture was cooled, diluted with water, and extracted with CH$_2$Cl$_2$ containing a small amount of ethyl acetate to effect solution of all solids. The organic phase was washed twice with water, dried (Na$_2$-SO$_4$), and stripped to a white solid. This was flash chromatographed on silica gel (75 g) (25 → 100% EtOAc in hexane + 1.5% acetic acid) to recover 7% of the starting material and 2.18 g (76%) of **2** as a white solid; mp 156–157.5 °C. TLC R_f 0.4 (EtOAc:hexane = 1:1, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 6.80–6.97 (m, 4 H, Ar), 7.18–7.63 (m, 4 H, Ar), 9.5 (bs, 1 H, OH), 13.1 (bs, 1 H, COOH).

1.3. Ethyl 3-(4-Hydroxyphenoxy)benzoate (3). A mixture of **2** (2.18 g, 9.47 mmol), anhydrous ethanol (35 mL), triethyl orthoformate (1.96 g, 13.2 mmol), and concentrated H$_2$SO$_4$ (0.5 mL) was heated under N$_2$ at 60–65 °C for 20 h and then poured into water and extracted with EtOAc/hexane. The organic phase was washed with NaHCO$_3$ solution and water, dried (Na$_2$SO$_4$), and stripped to an oil. This was flash chromatographed on silica gel (35 g) (20 → 30% EtOAc in hexane + 1.5% acetic acid).

Fractions containing product were combined, washed with NaHCO$_3$ solution, and stripped to recover 2.43 g (98%) of **3** as a colorless oil. TLC R_f 0.33 (EtOAc:hexane = 1:3, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 1.37 (t, J = 7.1 Hz, 3 H, OCH$_2$-CH$_3$), 4.36 (q, J = 7.1 Hz, 2 H, OCH$_2$CH$_3$), 5.25 (s, 1 H, OH), 6.82–6.95 (m, 4 H, Ar), 7.12–7.73 (m, 4 H, Ar).

1.4. Ethyl 3-(4-(2,2-Diethoxyethoxy)phenoxy)benzoate (4). A mixture of **3** (800 mg, 3.10 mmol), bromoacetaldehyde diethyl acetal (656 μL, ~1.4 X), anhydrous K$_2$CO$_3$ (0.96 g, 1.5 X), dimethyl formamide (6.4 mL), and KI (1–2 mg) was magnetically stirred under N$_2$ at 125 °C for 4.5 h. The cooled mixture was diluted with EtOAc (4 mL) and water (10 mL), and the aqueous phase was extracted with additional solvent. The combined organic extract was washed with water, dried (Na$_2$-SO$_4$), and stripped to a tan oil. This was flash chromatographed on silica gel (30 g), (5 → 30% EtOAc in hexane) to recover 268 mg (34%) of **3** and 618 mg (80% based on recovered **3**) of **4**. TLC R_f 0.37 (EtOAc:hexane = 1:4). ^{1}H NMR (CDCl$_3$): δ 1.26 (t, J = 7.0 Hz, 6 H, CH$_2$CH_3 acetal), 1.37 (t, J = 7.1 Hz, 3 H, CH$_2$CH_3 ester), 3.65 (dq, J = 7.0, 9.4 Hz, 2 H, HCHCH$_3$ acetal) 3.78 (dq, J = 7.1, 9.4 Hz, 2 H, HCHCH$_3$ acetal), 4.00 (d, J = 5.2 Hz, 2 H, CH$_2$OAr), 4.35 (q, J = 7.1 Hz, 2 H, CH$_2$CH$_3$ ester), 4.85 (t, J = 5.2 Hz, 1H, CH), 6.90–6.98 (m, 4 H, Ar), 7.10–7.74 (m, 4 H, Ar).

1.5. 3-(4-(2,2-Diethoxyethoxy)phenoxy)benzoic Acid (5). A mixture of **4** (400 mg, 1.15 mmol), 50% NaOH solution (940 mg, 4X), water (0.4 mL), 1,2-dimethoxyethane (glyme) (3.6 mL), and 40% Triton B in methanol (1 mL) was stirred under N$_2$ and heated at 75–80 °C for 10 h. The cooled solution was partially stripped of solvent, diluted with water (homogeneous), acidified to pH 3, and immediately extracted with EtOAc and water, washed, dried, and stripped to give 368 mg of white solid. This was recrystallized from hot butyl chloride with dilution with hexane to return 341 mg (92%) of **5**; mp 101.5–102.5 °C. TLC R_f 0.17 (EtOAc: hexane = 1:4, 1.5% acetic acid). ^{1}H NMR (CDCl$_3$): δ 1.26 (t, J = 7.0 Hz, 6 H, CH$_2$CH$_3$), 3.66 (dq, J = 7.1, 9.3 Hz, 2 H, HCHCH$_3$), 3.79 (dq, J = 9.3 Hz, 2 H, HCHCH$_3$), 4.01 (d, J − 5.2 Hz, 2 H, CH$_2$O), 4.85 (t, J = 5.2 Hz, 1 H, CH), 6.91–7.00 (m, 4 H, Ar), 7.17–7.79 (m, 4 H, Ar).

1.6. 3-((2-Oxoethoxy)ethoxy)phenoxybenzoic Acid (6). A solution of **5** (400 mg, 1.15 mmol) in glyme (4 mL) was treated with 3 N HCl (2 mL) and stirred under N$_2$ and heated at 75–80 °C for 30 min. The initial two phase reaction mixture became homogeneous after 5 min. Most of the solvents were stripped off under reduced pressure, and the residue was diluted with water and extracted with EtOAc. The extract was washed, dried (Na$_2$SO$_4$), and stripped. The gummy residue was flash chromatographed on 13 g of silica gel (10 → 70% EtOAc in hexane) to return 287 mg (90%) of the aldehyde (**6**) as a white solid, mp 143–147 °C, with a sintering from 139 °C. TLC R_f 0.56 (1.5% acetic acid in EtOAc). ^{1}H NMR (DMSO-d_6): δ 4.88 (s, 2 H, CH$_2$), 6.97–7.67 (m, 8 H, Ar), 9.7 (s, 1 H, CHO), 11.1 (bs, 1 H, COOH). FAB-MS m/z calculated for {M + H}$^+$ = C$_{15}$H$_{13}$O$_5$ 273, observed 273.

2. Synthesis of FPBA−Glycine. 2.1. Preparation of 3-Bromo-4-fluorobenzaldehyde (16). A solution of 4-fluorobenzaldehyde (2.48 g, 20 mmol) in dry methylene chloride (5 mL) was added to an ice-cooled suspension of powdered aluminum trichloride (2.66 g, 20 mmol) in dry CH$_2$Cl$_2$ (15 mL), in which bromine (3.4 g, 1.1 mL, 21.25 mmol) was added dropwise. The mixture was refluxed for 16 h at 45 °C, cooled, and carefully poured into ice H$_2$O and then extracted with CH$_2$Cl$_2$ (3 × 10 mL). The combined organic phase was washed sequentially with saturated sodium metabisulfite (20 mL) and H$_2$O (20 mL) and saturated with NaCl (20 mL) and then dried with magnesium sulfate. The resultant dark red oil, after removal of solvent under reduced pressure, was run on flash chromatography (hexane:EtOAc, 6:1) to give a sticky oil (1.60 g). Crystallization in the mixture (hexane:EtOAc, 20:1) gave the title compound (1.3 g) as a white solid. Yield: 32% (95% purity). R_f = 0.52 (hexane:EtOAc, 10:1). mp 30–32 °C. ^{1}H NMR (CDCl$_3$): δ 9.95 (s, 1H, CHO), 7.30–8.12 (m, 3H, Ar). m/z: 203 (M$^+$, 100).

2.2. Preparation of 3-Phenoxy-4-fluorobenzaldehyde. The title compound was prepared according to the procedure of Maurer (*17*), which included two steps: protection of the aldehyde and Williamson condensation. A mixture of 3-bromo-4-fluorobenzaldehyde (0.74 g, 5 mmol), triethyl orthoformate (0.83 mL, 5 mmol) and *p*-toluenesulfonic acid monohydrate (0.1 g), and absolute ethanol (20 mL) was refluxed overnight. A few pellets of sodium hydroxide were added, and then, the mixture was refluxed for 20 more minutes. After it was cooled, the reaction mixture was filtered and evaporated under reduced pressure to give a sticky cream. The cream was dissolved in 20 mL of ethyl ether and filtered through a pad of sodium sulfate. The ether was removed under reduced pressure. The resultant oil was dried overnight under vaccum and used in the next step.

Dimethylformate (10 mL) was distilled at 160 °C from a mixture of dimethylformate (60 mL) and phenol (0.7 g, 7.5 mmol). After the mixture was cooled, sodium hydride (0.32 g, 8 mmol) was added by portions and the reaction continued for 30 min at RT, and then, copper(I) chloride (0.2 g) was added. The reaction mixture was heated to 155 °C, and the protected aldehyde (5 mmol) was added over 10 min, and then, it was refluxed for 17 h at 155−165 °C. Subsequently, the reaction mixture was filtered, washed with 5% potassium carbonate, and extracted with petroleum ether (3 × 20 mL). Then, the ether layer was dried overnight with magnesium sulfate. After removal of solvent, the oil was deprotected in 10 mL of acetone containing hydrochloric acid (1.7 mL, 5.1 mmol). After removal of acetone, the residue was washed with 5% potassium carbonate and extracted with petroleum ether (3 × 20 mL), which was then dried with magnesium sulfate. Finally, after removal of the ether, the resultant oil was run on chromatography with a mobile phase (hexane:EtOAc, 20:1) to give the title compound (0.75 g). Yield: 69.4%, purity 92.3%. ^{1}H NMR: (CDCl$_3$): δ 9.92 (s, 1H, C*H*O), 6.89−7.53 (m, 8H, Ar). MS: *m/z* 216 (M$^+$, 100).

2.3. Preparation of FPBA (*18*). Sodium periodate (0.35 g, 1.6 mmol) was added to a mixture containing carbon tetrachloride (2 mL), acetonitrile (2 mL), water (4 mL), and 4-fluoro-3-phenoxy benzaldehyde (0.178 g, 0.8 mmol). The mixture was vigorously stirred while ruthenium chloride hydrate (0.15 g, 0.7 mmol) was added. The reaction was continued at RT until the starting material disappeared by TLC analysis. The reaction mixture was extracted with methylene chloride (3 × 10 mL). The combined organic phase was dried with magnesium sulfate, filtered, and evaporated under reduced pressure to give a brown oil. The oil was dissolved in diethyl ether and washed with 10% sodium hydroxide solution (2 × 10 mL). Acidification of the aqueous phase gave a white solid, which was reextracted with diethyl ether. The ether layer was washed with water and dried over magnesium sulfate, and the solvent was evaporated under reduced pressure to give the title compound (0.136 g) as a white solid. Yield: 71.2%. Purity: 96.4%. mp 99−100 °C. R_f = 0.39 (CHCl$_3$:CH$_3$OH:acetic acid, 20:1:0.1). ^{1}H NMR (DMSO-d_6): δ 7.05−7.77 (m, 8H, Ar), 12.30 (S, 1H, COO*H*). *m/z* ([M + 1]$^+$) = 232.04.

2.4. FPBAG. Thionyl chloride (0.41 g, 3.45 mmol) under N$_2$ protection was added to a mixture of FPBA (0.4 g, 1.72 mmol), chloroform (3 mL), and dimethylformate (0.5 mL). The mixture was heated to 60−65 °C for 1 h, and the solvent was stripped under reduced pressure. A few drops of toluene were added and then restripped under reduced pressure to give crude 4-fluoro-3-phenoxybenzoyl chloride. The crude product was added in portions to a vigorously stirred ice-cooled solution containing glycine (0.143 g, 1.9 mmol) and potassium hydroxide (3 mL, 2 N). Meanwhile, a potassium hydroxide solution (3 mL, 2 N) was added dropwise over 10 min to keep the aqueous phase slightly basic. The reaction mixture was acidified (3 N HCl) and extracted with diethyl ether. The combined ether was washed with water and evaporated to give a sticky oil. Chromatography (hexane:EtOAc, 1:1 → 0:1) of the oil gave the title compound (FPBAG) as a sticky oil (0.212 g). Purity: 90.1%. ^{1}H NMR (DMSO-d_6): δ 7.01−7.67 (m, 8 H, Ar), 8.01 (t, 1H, N*H*), 3.97 (d, 2H, C*H$_2$*), 12.10 (s, 1H, COO*H*). MS ([M + 1]$^+$) = 289.17.

Conjugation of Hapten 6-Thyroglobulin/BSA. The conjugate was synthesized using Schiff base formation and the reductive amination method (*19*). Fifty milligrams of thyroglobulin or BSA was dissolved in 10 mL of carbonate buffer (pH 9). Then, compound **6** (0.05 mmol) was added with gentle stirring followed by addition of 100 µL of 5 M cyanoborohydride in 1 N NaOH. The reaction mixture was stirred for 3.5 h at RT. The unreacted aldehyde sites were blocked by adding 200 µL of 3 M ethanolamine solution (pH adjusted to approximately 9 with 2 N HCl) and reacted for 15 min at RT. The solution was then dialyzed against PBS over 72 h at 4 °C and stored at −20 °C. The **6**−Thyr conjugate was used for immunization, and the **6**−BSA (cAg**01**) was used as a cAg.

Preparation of cAgs cAg 03, cAg 04, and cAg 05. The conjugate was synthesized using an activated ester method (*20*). Compounds 3-(4′-methoxyphenoxy)benzoic acid, 4-phenylbenzoic acid, or phenoxyacetic acid (0.025 mmol) were dissolved in 1.6 mL of dry DMF, and then, 6 mg (0.05 mmol) of NHS and 5.8 mg (0.03 mmol) of EDC were added. The reaction mixture was stirred overnight at RT. Forty milligrams of BSA was dissolved in 12 mL of PBS and 4 mL of carbonate buffer (pH 9). The activated hapten was added dropwise to the protein solution. The mixture was stirred for 30 min at RT and 7 h at 4 °C. The solution was then dialyzed against PBS over 72 h at 4 °C and stored at −20 °C.

Immunization. The production of Ab was made following the protocol reported earlier (*14*). Briefly, two New Zealand white rabbits were immunized (rabbit nos. 294 and 295) with **6**−Thyr. The antigen solutions (100 µg in PBS) were emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. After 1 month, the animals were boosted with an additional 100 µg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1 v/v). Booster injections were given at 4 week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C. The results of Ab characterization were obtained from sera of terminal bleeds after four boosters. These terminal bleeds were used for ELISA development.

ELISA. The competitive inhibition ELISA format in this study was based on methods described by Voller et al. (*21*). Microplates were coated overnight at 4 °C with 100 µL/well of the appropriate cAg concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After the plates were washed with PBST (PBST: 8 g/L NaCl, 1.15 g/L Na$_2$HPO$_4$, 0.2 g/L KCl, and 0.05% Tween-20), the plate was incubated with 200 µL per well of a 0.5% BSA solution in PBS for 30 min at RT. After another washing step, 100 µL per well of antiserum diluted in PBS per well (for titration experiment) or 50 µL/well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 µL/well of standard analyte or sample solution were added and incubated for 1 h. The standard analyte concentrations ranged from 0.05 to 5 µg/L. Following a washing step, the secondary Ab GAR-HRP (diluted 1:3000 in PBS with 0.05% Tween 20, 100 µL/well) was added and incubated for 1 h at RT. The plates were washed again, and 100 µL/well of substrate solution (3.3 µL of 30% H$_2$O$_2$, 400 µL of 0.6% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) was added. The color development was stopped after 15 min with 50 µL/well of 2 M H$_2$SO$_4$. The absorbance was measured using the dual wavelength mode at 450 nm minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$ where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is IC$_{50}$, and D is the minimum absorbance at infinite concentration.

Assay Optimization. The assay conditions were optimized in such a way that the IC$_{50}$ values were minimized. This goal was achieved by screening antibodies and antigens in a two-dimensional titration for best dilution of cAg and antiserum. Then, competitive inhibition curves were measured for different Ab and antigen combinations, and the one with the lowest IC$_{50}$ was selected for further assay development.

Cross-Reactivity (CR). The optimized assay was submitted to cross-reactivity studies by using the standard solution of the analytes and other structurally related compounds (listed in Table 3). The CR was obtained from the IC_{50} values of metabolite standard and the related compounds from the same plate where

$$\%CR = (IC_{50} \text{ of metabolite}/IC_{50} \text{ of tested compound}) \times 100$$

Matrix Effects. 1. pH. The pH effect was tested by preparing analytes in phosphate buffers at pH 4, 6, and 8.

2. Ionic Strength. The effect of ionic strength on the quantitation of PBA was studied by preparing analyte standard solutions in 0.1, 0.3, 0.5, and 0.7 M PBS all at pH 7.5.

3. Urine. The effects of urine matrix on the quantitation of PBA were evaluated by preparing analyte standard solutions in a buffer with different concentrations of urine (0, 1, 2, 4, or 10% of urine). Urine samples tested in this study were from healthy individuals without known exposure to pyrethroids.

Urine Sample Analysis. Urine samples collected from persons exposed to cyfluthrin were used in this study (provided by Dr. Gabriele Leng). In Dr. Leng's lab, these urine samples have been analyzed for the total FPBA after hydrolysis via GC-MS. To test which form of conjugate is predominant in the urine, these samples were tested by different immunoassays including the PBA−glycine ELISA (*14*). Each urine sample was split into three aliquots. One aliquot was analyzed directly by PBA and PBA−glycine ELISAs to determine the free FPBA and FPBA−glycine conjugate in urine. The second aliquot was incubated with glucuronidase and then measured with the PBA ELISA to determine the FPBA−glucuronide conjugate in urine (the difference in FPBA concentration measured before and after enzyme treatment). The third aliquot was hydrolyzed as described by Leng et al. (*22*) followed by the PBA ELISA to measure total free FPBA and FPBA conjugates in urine.

Results and Discussion

Hapten Design and Synthesis. No study has reported which conjugate is predominant for pyrethroid metabolites in human urine. The predominate conjugate may vary with an individual, exposure level, and other factors. In mammals, the major "detoxification" reaction for carboxylic acid-containing xenobiotics is conjugation, either with an amino acid to form a peptide or with glucuronic acid to form a glycoside (*23, 24*). Therefore, PBA, a main breakdown metabolite of pyrethroids, may undergo either amino acid conjugation or glucuronidation in humans. A specific assay for each possible conjugate may be ideal but not economically feasible for exposure monitoring. One alternative way is to hydrolyze all possible conjugates to free PBA and measure the total PBA in urine. This would allow monitoring of human exposure to total pyrethroids.

To generate a specific Ab for an analyte, it is important to maintain the structure of target compound with as little change as possible when it is modified and coupled to a carrier protein. In this respect, it is prudent to attach the handle as distal as possible from the structure that defines the target (including carboxylic acid group). The PBA molecule contains a carboxylic acid group, which can be directly conjugated to the carrier protein. However, such a strategy may result in antibodies with low sensitivity and high CR to parent pyrethroid molecules. A monoclonal Ab (from Shell Chemical Co.) generated using such a strategy was tested and its IC_{50} for PBA was about 3.0 ppm. This suggests that the carboxylic acid group might be important to develop a more sensitive and selective PBA assay. In the previous study for a PBA−glycine conjugate immunoassay, we successfully

Table 2. Selected Competitive ELISA Results for Screening from a Single Data Set

Ab/antigen	OD_{max} (A)	slope (B)	IC_{50} (C) (ng/mL)	OD_{min} (D)
Ab294/cAg**06**	0.89	1.16	1.28	0.06
Ab294/cAg**02**	0.95	1.33	2.83	0.06
Ab294/cAg**07**	0.85	0.96	5.78	0.06
Ab294/cAg**01**	0.95	1.10	121	0.06
Ab295/cAg**01**	0.65	1.00	145	0.07

developed a highly sensitive ELISA by designing a hapten with an attachment of a handle at the α-position of the glycine and leaving the carboxylic acid group unchanged. Similarly, in this study, haptens for PBA had the handle attached to the 4-position of distal phenyl group (Figure 2).

Screening of the Sera and Assay Optimization. A checkerboard titration system was used for screening of Ab and antigen combinations (*25*). The antisera of two rabbits were tested against seven cAgs. Both antisera showed higher titer in the homologous system than in heterologous systems. The main reason the titer is higher in the homologous system is that the identity of the hapten in the cAg and immunogen is identical. Recognition by both antibodies for the related biphenyl cAg or the partial molecule, phenoxyacetic acid, was poor suggesting that the PBA structure was required for recognition (Table 1). Ab 294 showed higher titer on all cAgs tested than Ab 295; thus, only Ab 294 was used for further screening.

All heterologous combinations tested showed a very low IC_{50} (<6.0 μg/L) against the target analyte, which ranged from 6.0 to 1.28 μg/L (Table 2). The homologous systems had higher IC_{50} values and were less desirable than the heterologous systems. For example, with Ab 294, the IC_{50} for PBA in a homologous system was about 50 times higher than that in the heterologous systems (Table 2). Of the cAgs screened, the BSA conjugate of PBA (cAg**06**) yielded the most sensitive assay. In this study, only Ab 294/cAg**06** was used for further assay development.

CR. Esfenvalerate, cypermethrin, permethrin, deltamethrin, cyfluthrin, their metabolites, and other structurally related compounds were tested for CRs (Table 3). Ab 294 is highly selective for the target analyte PBA and the related cyfluthrin metabolite FPBA (72%). In all cases, the CR of the parent compound, metabolites, and other tested compounds was negligible. Although PBA−glycine, PB alcohol, and other tested pyrethroids contain the PB group, which is present in the immunogen hapten, they did not interfere in the assay.

Matrix Effects. Because this assay was intended to analyze urine samples, it was necessary to study the influence of pH and ionic strength in urine samples. This assay showed good tolerance to the pH of the solution (Figure 4). As compared with PBS buffer (0.15 M, pH 7.5), no significant influences were observed at pH 5−8 indicating that a slight difference in the pH of sample or buffer (in this range) would not affect the accuracy of PBA quantitation.

The assay is very sensitive to higher concentrations of salt in solution (Figure 5). At an ionic strength of 0.3 M PBS, the binding between Ab and antigen was suppressed about 30%. These results are consistent with the previous report about atrazine mercapturic acid and assumed that there was a disruption of Ab−antigen interaction (*26*).

Table 3. Summary of Cross-Reactivities of PBA Assay

Chemicals	Structures	IC$_{50}$ (ng/mL)	CR (%)
3-Phenoxybenzoic acid (PBA)		1.65	100
4-Fluoro 3-phenoxybenzoic acid (FPBA)		2.28	72
PBA-glycine conjugate (PBAG)		>10000	0
FPBA-glycine conjugate (FPBAG)		>10000	0
3-Phenoxybenzyl alcohol		>10000	0
4-Hydroxybenzoic acid		>10000	0
Permethrin		>10000	0
Cypermethrin		>10000	0
Esfenvalerate		>10000	0
Deltamethrin		>10000	0
Cyfluthrin		>10000	0

The effect of a urine matrix was evaluated as well. In tests with four different urine samples, this assay could tolerate 2% of urine with little alteration in the standard curve (Figure 6). Therefore, dilution prior to ELISA would be necessary for the assay. Considering variation among different urine samples, a 100× dilution of the urine before the assay was chosen. Alternatively, differential partitioning or solid phase extraction could be used to clean up the sample. In recovery experiments for the direct dilution of urine samples, urine matrix had a little effect on accurate quantitation for urines spiked with PBA ≥ 5.0 μg/L. The recovery rate for PBA at 5.0 μg/L is 110.0 ± 10.5%. According to the recommended LOQ determination guideline (*27*), the approximate LOQ was 5.0 μg/L for the assay with no clean up. This is consistent with the estimated concentration that corresponded to the absorbance of the control (zero analyte) minus three times the standard deviation of control, when standard analytes were prepared in a buffer system with 2% blank urine.

Assay Validation and Application. The assay validation was performed in a blind fashion by direct dilution
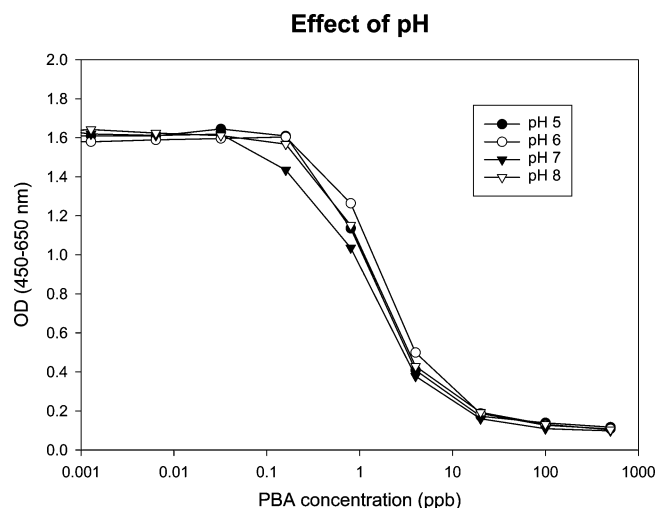

Figure 4. ELISA competition curves of PBA prepared at various pH values. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).
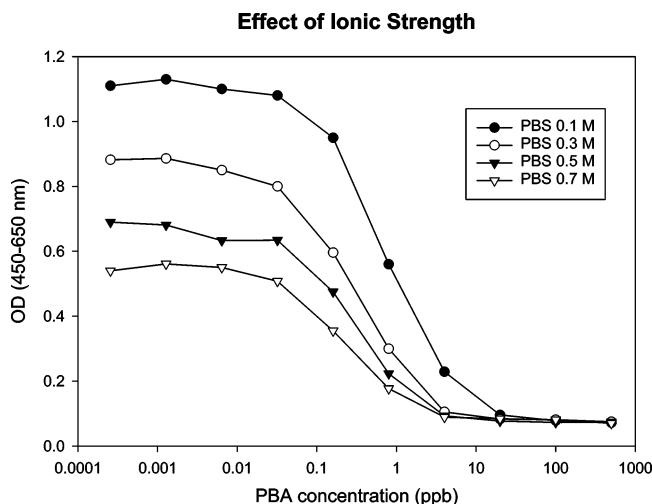
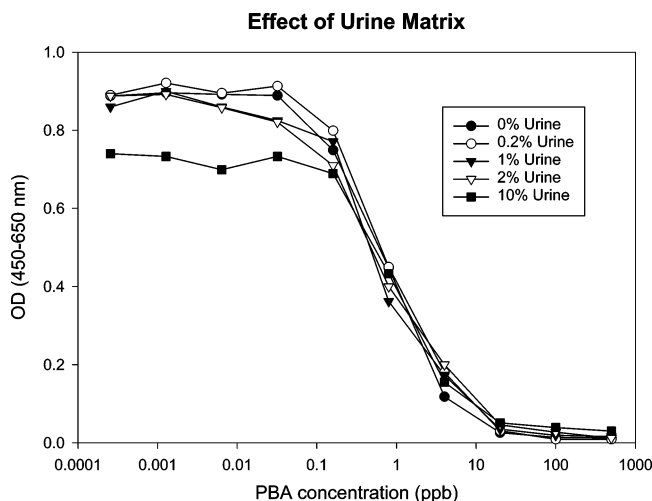**Effect of Ionic Strength**

Figure 5. ELISA competition curves of PBA prepared at various ionic strengths. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).

Effect of Urine Matrix

Figure 6. ELISA competition curves of PBA prepared at various urine concentrations. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).

of the urine samples, which were spiked with PBA concentrations ranging from 0 to 160 μg/L (Figure 7). The linear regression analysis of ELISA results showed a good correlation ($R^2 = 0.900$). All recoveries were over 86% of the spiked values. These results demonstrated that these assays are suitable for the quantitative detection of pyrethroid metabolite (as total PBA) at trace levels in urine samples.

Two positive human urine samples (I and II) from the laboratory of Dr. Gabriele Leng were tested using the PBA ELISA and the PBA–glycine ELISA (*14*). In the PBA–glycine assay, PBA–glycine was used as reference standard. On the basis of the structural similarity between PBA and FPBA (Table 4) and high CR of FPBA in the PBA assay (72%), the FPBA–glycine should be highly cross-reactive in the PBA–glycine assay. No FPBA–glycine was detected (<2.0 ng/mL as PBA–glycine) in either urine sample. A moderate amount of FPBA–glucuronide was detected in these urine samples. This conjugate is about 7.2 (8.4 ng/mL) and 6.2% (2.4 ng/mL) of the total FPBA in urine samples I and II, respectively. Interestingly, the major FPBA-containing

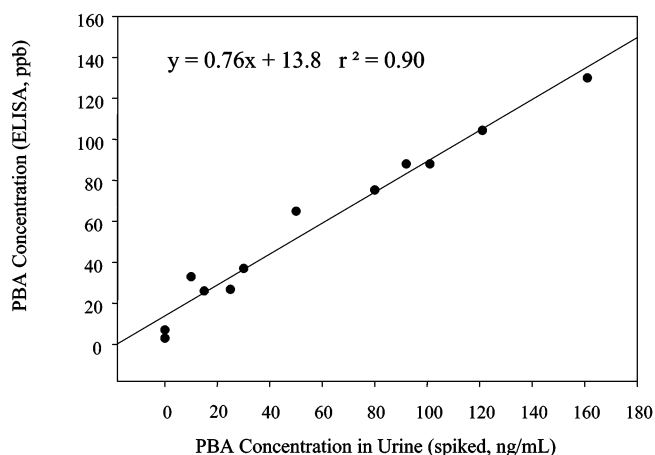**Relationship between PBA Spiked into Urine and Determined by ELISA**

Figure 7. Relationship between spiked PBA in urine and measured by ELISA. Blank urine samples were provided by lab personnel without known exposure to pyrethroids.

Table 4. Summary of Cyfluthrin Metabolites in Human Urine

| | prehydrolysis | | | | posthydrolysis | |
| | | | | | FPBA (ng/mL) | |
	FPBAG (ng/mL)	glucuronide (ng/mL)	FPBA (ng/mL)	others (ng/mL)	ELISA	GC-MS
urine I	ND[a]	8.4	68.7	20.5	117	97.6
urine II	ND	2.4	28.2	4.0	39	34.6

[a] ND: not detectable at the detection limit of PBA-glycine ELISA (5 ng/mL).

Standard Curve of PBA Immunoassay

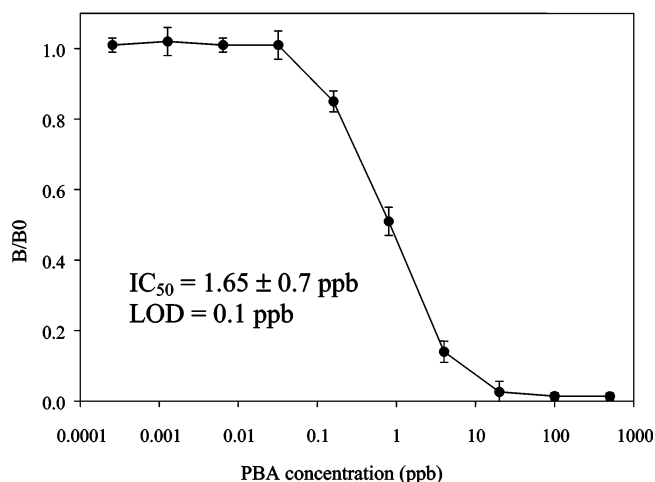$$IC_{50} = 1.65 \pm 0.7 \text{ ppb}$$
$$LOD = 0.1 \text{ ppb}$$

Figure 8. ELISA inhibition curve for PBA using antiserum 294 (diluted 1:10 000, final dilution in well), cAg PBA-BSA (cAg**06**) (0.5 μg/mL), and GAR-HRP (1:3000). This standard curve represents the average of 23 curves.

metabolite in these urine samples is free FPBA, which was 59 (68.7 ng/mL) and 72% (28.2 ng/mL) of the total FPBA metabolites in urine samples I and II, respectively. This may be due to the degradation of FPBA conjugates during storage because the urine samples were stored at 0–4 °C for 3–4 months prior to this study. A further study using freshly collected urine samples is needed to determine whether free FPBA or PBA is the predominant form of pyrethroid metabolite in urine. If confirmed, PBA or FPBA can be used directly as a biomarker of human exposure to pyrethroids. A good agreement between ELISA and GC-MS results was observed (Table 4)

suggesting that this assay is suitable for human urine monitoring.

Conclusions. A sensitive and selective immunoassay for PBA has been developed by using **6** as the immunizing hapten and PBA as the cAg hapten. ELISA cAg**06**/294 has a very low IC_{50} value and also exhibits good performance characteristics at various pH values. The assay is sensitive to urine matrix and a $50\times$ dilution is needed before analysis. The optimized assay using antiserum 294 (diluted 1:10 000, final dilution in well) and cAg**06** (0.5 μg/L) gave an IC_{50} value of 1.65 ± 0.7 ng/mL with a lower detection limit of 0.1 ng/mL (Figure 8). This ELISA was successfully applied to quantitate low parts per billion (ppb or ng/mL) of PBA in urine. A good correlation between ELISA and GC-MS results was achieved in the samples from exposed workers suggesting that this PBA immunoassay is useful for human exposure monitoring and toxicological studies.

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