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 IC50 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 PB1 group and PBA is a common metabolite form or intermediate, PBA conjugates might be suitable biomarkers of human exposure.

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; IC50, 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.
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, fluvinate, fenvalerate, and permethrin were obtained from Riedel de Haen (Seelze, Germany). Esfivalerate 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-Methoxyphenoxyl)-benzoic Acid (1).** 3-(4-Methoxyphenoxyl)benzaldehyde (5.0 g) was mixed with 50 mL of distilled water containing 1.0 g of K2CO3. At RT, a catalytic amount of tetrabutylammonium chloride was added, and then, 1.0 g of KMnO4 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 NaHSO3 was added and stirred for 15 min to remove unreacted KMnO4. 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 4.0 g of

<table>
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<th>Coating Antigens</th>
<th>Structures</th>
<th>Ab 294</th>
<th>Ab 295</th>
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| cAg 01 Compound 6-BSA | ![Structure](image) | ++++ | ++++
| cAg 02 PBA-glycine-BSA | ![Structure](image) | ++++ | +
| cAg 03 4-methoxy-PBA-BSA | ![Structure](image) | + | -
| cAg 04 4-biphenyl benzoic acid-BSA | ![Structure](image) | - | -
| cAg 05 phenoxyacetic acid-BSA | ![Structure](image) | - | -
| cAg 06 3-PBA-BSA | ![Structure](image) | ++++ | +
| cAg 07 N-(3-Phenoxymethyl)-4-amino-L-phenylalanine-BSA | ![Structure](image) | ++++ | +

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 0.75–1.00; and ++++, absorbance > 1.00.
Fractions containing product were combined, washed with NaHCO₃ solution, and stripped to recover 2.43 g (98%) of 3 as a colorless oil. TLC Rₚ 0.33 (EtoAc:hexane = 1:3, 1.5% acetic acid). ¹H NMR (DMSO-d₆): δ 1.37 (t, J = 7.1 Hz, 3 H, OCH₂CH₃), 4.36 (q, J = 7.1 Hz, 2 H, OCH₂CH₂CO₂H), 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-Diethoxyethoxy)phenoxy)benzoate (4). A mixture of 3 (800 mg, 3.10 mmol), bromoacetalddehyde diethyl acetal (656 μL, ~1.4 X), anhydrous K₂CO₃ (0.96 g, 1.5 X), dimethyl formamide (6.4 mL), and KI (1–2 mg) was magnetically stirred under N₂ at 125 °C for 4.5 h. The cooled solution 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 (Na₂SO₄), and stripped to a tan oil. This was flash chromatographed on silica gel (90 g) (4.5% EtOAc in hexane) to recover 268 mg (34%) of 3 and 618 mg (80% based on recovered 3) of 4. TLC Rₚ 0.37 (EtoAc:hexane = 1:4). ¹H NMR (CDCl₃): δ 1.26 (t, J = 7.0 Hz, 6 H, CH₂(CH₂)₃ acet), 1.37 (t, J = 7.1 Hz, 3 H, CH₂CH₃ ester), 3.65 (dq, J = 7.0, 9.4 Hz, 2 H, HCH₂CH₂ acet) 3.78 (dq, J = 7.1, 9.4 Hz, 2 H, HCH₂CH₂ acet). 4.00 (d, J = 5.2 Hz, 2 H, CH₂OAr), 4.35 (q, J = 7.1 Hz, 2 H, CH₂CH₂ ester). 4.85 (t, J = 5.2 Hz, 1 H, 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, 40X), water (0.4 mL), 1,2-dimethoxyethane (glyme) (3.6 mL), and 40% Triton B in methanol (1 mL) was stirred under N₂ 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:EtOAc (20:1) to recover 287 mg (90%) of the aldehyde (3). TLC Rₚ 0.39 (chro:hexane = 1:1, 1.5% acetic acid). ¹H NMR (CDCl₃): δ 1.25 (t, J = 7.0 Hz, 6 H, CH₂(CH₂)₃ acet), 1.37 (t, J = 7.1 Hz, 3 H, CH₂CH₃ ester), 3.65 (dq, J = 7.0, 9.4 Hz, 2 H, HCH₂CH₂ acet) 3.78 (dq, J = 7.1, 9.4 Hz, 2 H, HCH₂CH₂ acet). 4.01 (d, J = 5.2 Hz, 2 H, CH₂OAr), 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)phenoxy)benzoic 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 N₂ 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 (Na₂SO₄), 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 Rₚ 0.56 (1.5% acetic acid in EtoAc). ¹H NMR (DMSO-d₆): δ 3.68 (s, 2 H, CH₂), 6.97–7.67 (m, 8 H, Ar), 9.7 (s, 1 H, CHO), 11.1 (bs, 1 H, COOH).

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 CH₂Cl₂ (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 H₂O and then extracted with CH₂Cl₂ (3 × 10 mL). The combined organic phase was washed sequentially with saturated sodium metabisulfite (20 mL) and H₂O (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, 2:1) gave the title compound (1.3 g) as a white solid. Yield: 32% (95% purity). Rₚ = 0.52 (hexane:EtoAc, 10:1). mp 30–32 °C. ¹H NMR (CDCl₃): δ 9.95 (s, 1H, CHO), 7.30–8.12 (m, 3H, Ar), m/z: 203 (M⁺, 100).

Figure 2. Scheme for synthesis of hapten 6.

Figure 3. Scheme for synthesis of FPBA-glycine.

compound 1. TLC Rₚ 0.49 (ethyl acetate:hexane = 1:1, 1.5% acetic acid). ¹H NMR (DMSO-d₆): δ 3.77 (S, 3H, CH₃O), 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 xylene (1.5 mL) was refluxed for 1.2 h. The mixture was cooled, diluted with water, and extracted with CH₂Cl₂ containing a small amount of ethyl acetate to effect solution of all solids. The organic phase was washed twice with water, dried (Na₂SO₄), and stripped to a white solid. This was flash chromatographed on silica gel (75 g) (25% 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 Rₚ 0.4 (EtoAc:hexane = 1:1, 1.5% acetic acid). ¹H NMR (DMSO-d₆): δ 6.80–6.97 (m, 4 H, Ar), 7.38–7.63 (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 H₂SO₄ (0.5 mL) was heated under N₂ at 60–65 °C for 20 h and then poured into water and extracted with EtoAc:hexane. The organic phase was washed with NaHCO₃ solution and water, dried (Na₂SO₄), and stripped to an oil. This was flash chromatographed on silica gel (35 g) (20 → 30% EtoAc in hexane + 1.5% acetic acid).
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 oil. The oil was dried overnight under vacuum 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 hydroxide (0.32 g, 8 mmol) was added as 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 reflused 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 over magnesium sulfate, and the solvent was evaporated under reduced pressure. The resultant oil was dried overnight under vacuum and used in the next step.

After the mixture was cooled, sodium hydride (0.32 g, 8 mmol) was added to a mixture containing carbon tetrachloride (2 mL), acetonitrile (2 mL), water (4 mL), and 4-fluoro-3-phenoxybenzoyl chloride. The crude product was added in portions to a vigorously stirred ice-cooled solution containing 3-methoxyphenoxy)benzoic acid, 4-phenylbenzoic acid, or phenoxycetic 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 PBS (PBST: 8 g/L NaCl, 1.15 g/L Na2HPO4, 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 diluent 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 Ag 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% H2O2, 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 10 min with 50 μL/well of 2 M H2SO4. 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: 

\[
y = \frac{D}{1 + \left(\frac{A}{C}\right)^{-B}}
\]

where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is the IC50, and D is the minimum absorbance at infinite concentration.

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 best dilution of cAg and antisera. Then, competitive inhibition curves were measured for different Ab and antigen combinations, and the one with the lowest IC50 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 analyte and other structurally related compounds (listed in Table 3). The CR was obtained from the IC50 values of metabolite standard and the related compounds from the same plate where

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

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, 0.2, 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 predominant 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 glucoside (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 IC50 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 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, haptons 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 antiserum of two rabbits was 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 IC50 (<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 IC50 values and were less desirable than the heterologous systems. For example, with Ab 294, the IC50 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 (cAg06) yielded the most sensitive assay. In this study, only Ab 294/cAg06 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 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).
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-fold 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 at 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

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<th>Structures</th>
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<td><img src="image" alt="Structure" /></td>
<td>&gt;10000</td>
<td>0</td>
</tr>
<tr>
<td>Cyfluthrin</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt;10000</td>
<td>0</td>
</tr>
</tbody>
</table>

**Effect of pH**

Figure 4. ELISA competition curves of PBA prepared at various pH values. Reagent concentrations: cAg (cAg06, 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 ng/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 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).

<table>
<thead>
<tr>
<th>FPBAGlucuronide (ng/mL)</th>
<th>FPBA (ng/mL)</th>
<th>Others (ng/mL)</th>
<th>ELISA</th>
<th>GC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>urine I</td>
<td>ND</td>
<td>8.4</td>
<td>68.7</td>
<td>20.5</td>
</tr>
<tr>
<td>urine II</td>
<td>ND</td>
<td>2.4</td>
<td>28.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

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

Table 4. Summary of Cyfluthrin Metabolites in Human Urine
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 cAg06/294 has a very low IC₅₀ value and also exhibits good performance characteristics at various pH values. The assay is sensitive to urine matrix and a 50× dilution is needed before analysis. The optimized assay using anti-serum 294 (diluted 1:10 000, final dilution in well) and cAg06 (0.5 μg/L) gave an IC₅₀ 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 assay is suitable for human urine monitoring and toxicological studies.

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References


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