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Articles

An Enzyme-Linked Immunosorbent Assay for the Detection of Esfenvalerate Metabolites in Human Urine

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The pyrethroids are one of the most heavily used insecticide classes in the world. Sensitive and rapid analytical techniques are needed for assessments of human exposure to these compounds. Highly sensitive and selective ELISAs for glycine conjugates of esfenvalerate key metabolites phenoxybenzoic acid (PBA) and *s*-fenvalerate acid (sFA) were developed. Rabbits were immunized with either *N*-(3-phenoxybenzoyl)-4-amino-L-phenylalanine−fetuin or *N*-[(*S*)-4-chloro-2-(methylethyl)benzeneacetyl]-4-amino-L-phenylalanine−fetuin, and all sera were screened against numerous coating antigens. The antibodies with the least interference and best sensitivity were optimized and characterized. The I_{50}s for sFA-glycine and PBA-glycine in buffer were found to be 0.40 ± 0.12 μg/L (1.47 ± 0.44 nmol/L) and 0.42 ± 0.18 μg/L (1.56 ± 0.67 nmol/L), respectively. Both assays exhibited high selectivity. Little or no cross reactivity to the parent compound and other metabolites was measured. The matrix effects of urine were investigated. Solid-phase extraction (SPE) strategies were used in an attempt to decrease the matrix effects and increase the sensitivity of the overall method. The limit of quantitation (LOQ) for both sFA-glycine and PBA-glycine in urine with SPE is 1.0 μg/L (3.70 nmol/L). These assays could be used as markers of exposure for monitoring biological samples.

Introduction

Pyrethroids such as esfenvalerate, cypermethrin, and deltamethrin, a group of highly potent insecticides with relatively low mammalian toxicity, have been widely used in agriculture, forestry, horticulture, public health, and households all around the world (*1*−*3*). These neurotoxins act on the axons in the peripheral and central nervous systems by interacting with sodium channels in mammals and insects. A prolonged opening of the sodium channel immediately evokes a repetitive nerve action and causes a series of neurotoxic effects such as hyperactivity, tremor, ataxia, convulsion, and possible paralysis (*4*−*6*). Production personnel and farmworkers are exposed to pyrethroids during manufacture and application. Although these compounds are claimed to be safe for humans (*7*), after exposure, reversible symptoms of poisoning such as headaches, dizziness, nausea, irritation

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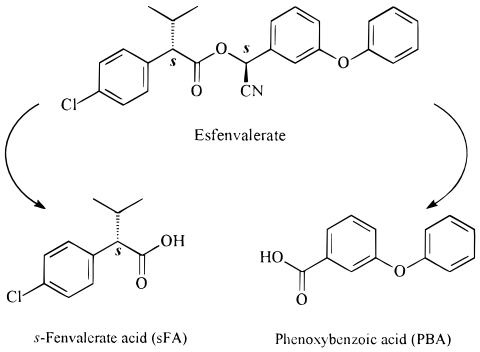


Figure 1. Structures of esfenvalerate and its metabolites.

of the skin and nose, and paraesthesia have been reported (*8*, *9*). Moreover, the chronic toxic effects in humans are not clear yet. Studies on chronic exposure effects by Hallenbeck and Cunningham-Burns (*10*) have suggested that fenvalerate may have caused lymph node and spleen damage as well as carcinogenisis. Recently, Repetto (*11*) demonstrated that pyrethroids have suppressive effects on the immune system. In studies on the estrogenic potential of pyrethroids in human breast carcinoma cell, Go et al. (*12*) suggest that pyrethroids should be considered hormone disruptors. Therefore, it is desirable that sensitive and efficient analytical methods be developed for both toxicological and epidemiological monitoring (*13*).

Studies of pyrethroid metabolism in mammals have revealed that pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage, followed by conjugation (*14*−*17*). Fenvalerate was shown to be metabolized in mammals to yield fenvalerate acid (FA)[1] and 3-phenoxybenzoic acid (PBA), which are conjugated in part to glucuronic acid and glycine, and then excreted renally (Figure 1) (*3*, *18*−*21*). Although no studies regarding the conjugates of pyrethroid metabolites in humans have been reported, it has been well established that conjugation with one of a variety of amino acids (such as glycine) is an important biotransformation pathway for xenobiotics containing a carboxylic acid group (*22*, *23*).

Current analytical methods for pyrethroid metabolites in urine rely upon multistep sample cleanup procedures, including hydrolysis, extraction, and derivatization. The samples are then analyzed using either HPLC (*20*) or gas chromatography with mass spectrometry (*20*, *24*−*27*). These methods are time-consuming and expensive, and often do not fulfill the requirements of routine analysis regarding sensitivity and practicality for effective monitoring. A promising method for rapid assessment of exposure to agrochemicals is the use of immunoassays that detect key urinary biomarkers of exposure (*28*). It has been demonstrated that immunoassays are highly sensitive and selective analytical tools for identifying trace chemicals such as pesticide residues and their

[1] Abbreviations: Ab, antibody; Ag, antigen; BSA, bovine serum albumin; cAg, coating antigen; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; FA, fenvalerate acid; sFA, esfenvalerate acid; FAB-HRMS, fast atom bombardment high-resolution mass spectroscopy; GAR−HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; I_{50}, concentration of analyte giving 50% inhibition; LOQ, limit of quantitation; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; PB, phenoxybenzyl; PBA, phenoxybenzoic acid; PBS, phosphate-buffered saline; PBST, phosphate buffered saline with 0.05% Tween 20; SPE, solid-phase extraction; TMB, tetramethylbenzidine.

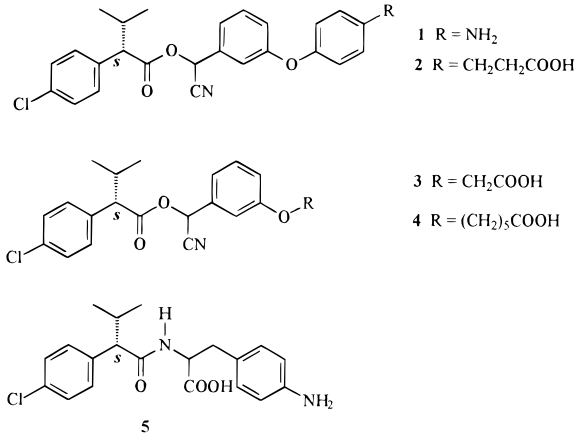


Figure 2. Structures of haptens **1**−**5**.

metabolites (*29*). Recently, several immunoassays have been developed for identifying pesticide metabolites as biomarkers of exposure, such as hydroxytriazines and dealkylated triazines (*30*), naphthalene mercapturates (*31*), and triazine mercapturates (*32*).

In the study presented here, we describe the development of immunoassays for the detection of glycine conjugates of the esfenvalerate metabolites PBA and sFA, which might be used as biomarkers of human exposure to esfenvalerate. The interference of urine as a matrix and the sample preparation method were investigated and will be discussed.

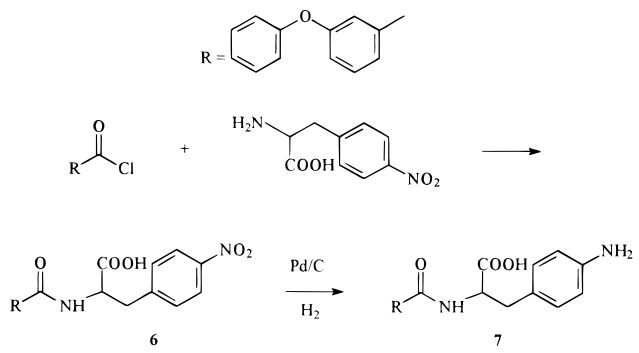
Experimental Procedures

Chemicals. The standards cypermethrin, fluvalinate, fenvalerate, and permethrin were obtained from Riedel de Haen (Seelze, Germany). Racemic 4-chloro-α-(1-methylethyl)benzeneacetic acid was generously supplied by E. I. duPont De Nemours & Co. (Wilmington, DE) and resolved to give the (*R*)- and (*S*)-isomers as described previously (*33*). Esfenvalerate was synthesized as described by Shan et al. (*33*) with a purity of >99% based on analytical data. Bovine serum albumin (BSA), ovalbumin (OVA), fetuin, Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), and Lancaster Synthesis, Inc. (Windham, NH). TLC was performed on 0.2 μm silica gel 60-F254 glass plates from E. Merck (Darmstadt, Germany). Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel, packed in glass columns with a diameter that gives a column height/diameter ratio of ∼7. The → notation denotes a stepwise solvent gradient.

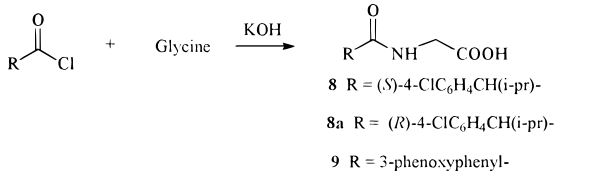
Instruments. NMR data were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million downfield from internal tetramethylsilane. Melting points were determined on a Thomas-Hoover Uni-Melt apparatus (Thomas Scientific, Swedesborogh, NJ) and are uncorrected. Fast atom bombardment high-resolution mass spectra (FAB-HRMS) were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.), using xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol or glycerol as the matrix. Polyethylene glycol was added to the matrix as a mass calibrant. ELISAs were carried out with 96-well microtiter plates and read with a microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Synthesis and Verification. Syntheses of the haptens were carried out as outlined in Schemes 1 and 2. All

Scheme 1



Scheme 2



reactions were straightforward, and yields were moderate to good. The syntheses of haptens **1**−**5** (Figure 2) are outlined in the previous paper describing an immunoassay for esfenvalerate (*33*).

***N*-(3-Phenoxybenzoyl)-4-nitro-L-phenylalanine (6).** 3-Phenoxybenzoic acid (1.48 g, 6.9 mmol) in 3 mL of $CHCl_3$ was treated under N_2 with $SOCl_2$ (0.73 mL, 10 mmol) and DMF (0.5 μL). After heating at 60−65 °C for 1 h, stripping, addition of hexane, and restripping, the acid chloride was obtained as a pale yellow liquid. This was added dropwise over the course of a few minutes to a vigorously stirred solution of 4-nitro-L-phenylalanine (1.5 g, 6.57 mmol) and potassium carbonate (1 g, 7.2 mmol) in 10 mL of water followed by dropwise titration with 10% sodium hydroxide over the course of 10 min to pH 9. After 10 min, the odor of acid chloride was no longer detectable. The mixture was acidified (3 N HCl) and extracted with ethyl acetate, and the organic phase was filtered, washed with water, and stripped to a yellow gum. Flash chromatography on 35 g of silica gel (CH_2Cl_2 → EtOAc → 3% HOAc/EtOAc) followed by crystallization from toluene gave 1.34 g (48%) of white solid: $[\alpha]_D^{24}$ −38.6 (*c* 0.755, EtOH); mp 140−146 °C. The hot melt recrystallized and remelted at 164−165 °C. The TLC of the melt [R_f = 0.45 (3% HOAc/THF)] was unchanged: ^{1}H NMR ($CDCl_3$) δ 3.32 (dd, J = 13.9, 5.9 Hz, 1 H, HCH), 3.48 (dd, J = 13.9, 5.8 Hz, 1 H, HCH), 5.09 (dt, J = 7.1, 5.8 Hz, 1 H, CHCOOH), 6.63 (d, J = 7.2 Hz, 1 H, NH), 6.99−8.13 (m, 13 H, Ar); ^{13}C NMR (DMSO) δ 36.0, 53.6, 117.1, 118.9 (2C), 121.6, 122.3, 123.2 (2C), 123.9, 130.1, 130.1 (2C), 130.4 (2C), 135.6, 146.3, 146.5, 156.2, 156.6, 156.8, 172.6.

***N*-(3-Phenoxybenzoyl)-4-amino-L-phenylalanine (7).** A solution of **6** (0.522 g, 1.28 mmol) and 10% palladium−carbon catalyst (4.5 mg) in 95% ethanol (20 mL) was stirred under hydrogen at 50 °C for 2.5 h and 25 °C for 3 h. The resulting suspension was diluted to 100 mL with ethanol, heated to 75 °C to dissolve solids, and filtered hot through Celite. Dilution with 60 mL of hexane and standing overnight gave 0.366 g (76%) of a white solid (mp 217−218 °C). A second crop of 92 mg (19%, mp 214−216 °C) was obtained from the filtrate: $[\alpha]_D^{24}$ (combined solids) −54 (*c* 1.08, DMF); TLC R_f = 0.16 (2.5% HOAc in 15:85 CH_2Cl_2/EtOAc); ^{1}H NMR (DMSO-d_6) δ 2.84 (dd, J = 13.6, 10.6 Hz, 1 H, HCHAr), 2.97 (dd, J = 13.7, 4.4 Hz, 1 H, HCHAr), 4.44 [m, J = 7.9, 4.4 Hz, 1 H, HCC(O)], 6.41−7.6 (m, 13 H, Ar), 8.63 (d, J = 8.0 Hz, 1 H, NH); ^{13}C NMR (DMSO-d_6) δ 35.9 (CH_2Ar), 55.2 (CHCOOH), 114.2 (2C), 117.6, 119.1 (2C), 121.7, 122.6, 124.1, 125.2, 129.8 (2C), 130.3, 130.4 (2C), 136.2, 147.2, 156.6, 157.0, 165.9 [C(O)N], 173.7 (COOH); FAB-HRMS *m*/*z* calcd for $C_{22}H_{21}N_2O_4$ ([M + H]$^+$) 377.1501, found 377.1520.

***N*-[(*S*)-4-Chloro-α-(1-methylethyl)benzeneacetyl]glycine (8).** (*S*)-4-Chloro-α-(1-methylethyl)benzeneacetyl chloride was prepared from the corresponding acid (2.12 g, 0.01 M) by the same procedure as described above for phenoxybenzoyl chloride. This was added dropwise over 10 min to a vigorously stirred ice-cooled solution of glycine (1.5 g, 0.02 M) and 4-(dimethylamino)pyridine (4 mg) in 10 mL of 2 N KOH. After an additional 15 min, the reaction mixture was neutral. The ice bath was removed, and additional base solution (4.5 mL) was added dropwise over the course of 10 min to keep the mixture slightly basic. After an additional 15 min, the reaction mixture was filtered through Celite to remove a small amount of flocculant precipitate, acidified (3 N HCl), and extracted with CH_2Cl_2. The extracts were washed with water, dried over $MgSO_4$, and stripped to a colorless gum. The residue was chromatographed on 40 g of silica gel (CH_2Cl_2 → 3% HOAc in EtOAc). Stripping product fractions gave a gum containing some acetic acid, which showed only one spot by TLC: R_f = 0.46 (3% HOAc in EtOAc). Crystallization from 6 mL of *n*-butyl chloride diluted with 2 mL of hexane gave 1.76 g (65%) of fine white needles (mp 137−139 °C). A second crop of 102 mg (mp 136−138 °C) was obtained from the concentrated filtrate: $[\alpha]_D^{24}$ (combined solids) 29.1 (*c* 1.17 $CHCl_3$); ^{1}H NMR ($CHCl_3$) δ 0.70 (d, J = 6.7 Hz, 3 H, CH_3), 1.03 (d, J = 6.5 Hz, 3 H, CH_3), 2.35 (m, J = 6.5, 10.3 Hz, 1 H, Me_2CH), 2.93 (d, J = 10.3 Hz, 1 H, Me_2CHCH), 3.88 (dd, J = 5.0, 18.5 Hz, 1 H, HCHCO), 4.08 (dd, J = 5.4, 18.5 Hz, 1 H, HCHCO), 6.38 (t, J = 5.0 Hz, 1 H, NH), 7.26 (s, 4 H, Ar); ^{13}C NMR (DMSO-d_6) δ 20.2 (CH_3), 21.1 (CH_3), 31.1 (Me_2C), 40.7, 58.7, 128.0 (2C), 130.0 (2C), 131.3, 139.0, 171.2, 172.9; FAB-HRMS *m*/*z* calcd for $C_{13}H_{17}ClNO_3$ ([M + H]$^+$) 270.09, found 270.09.

***N*-(3-Phenoxybenzoyl)glycine (9).** Crude stripped 3-phenoxybenzoyl chloride, prepared as described above from the acid (1.5 g, 7 mmol), was added in portions to a vigorously stirred ice-cooled solution of glycine (0.578 g, 7.7 mmol) in 7 mL of 1 N KOH along with equivalent portions of 2 N KOH over the course of 30 min. Additional caustic was added over the course of 15 min to keep the aqueous phase slightly basic. The homogeneous mixture was acidified (3 N HCl) and extracted with ether. The combined extract was washed with water and stripped to pale green oil. Crystallization from EtOAc/*n*-butyl chloride gave 0.88 g (46%) of a white solid (mp 148−150 °C). Chromatography of the filtrate (silica gel, CH_2Cl_2 → EtOAc) recovered an additional 8%: TLC R_f = 0.6 (3% HOAc in EtOAc); ^{1}H NMR (DMSO-d_6) δ 3.88 (d, J = 5.8 Hz, 2 H, CH_2), 7.03−7.67 (m, 9 H, Ar), 8.89 (t, J = 5.8 Hz, 1 H, NH), 12.6 (s, 1 H, COOH); ^{13}C NMR (DMSO-d_6) δ 41.4 (CH_2), 117.3, 118.9 (2C), 121.7, 122.4, 123.8, 130.2 (3C), 135.9, 156.4, 156.9, 165.8 [C(O)N], 171.3 [C(O)]; FAB-HRMS *m*/*z* calcd for $C_{15}H_{14}NO_4$ ([M + H]$^+$) 272.09, found 272.09.

Hapten Conjugation. Conjugates were synthesized using a diazotization, activated ester method and the periodate method (*34*). To obtain immunogens, haptens **5** and **7** were conjugated to fetuin. Coating antigens were made by coupling haptens **1**−**5** and **7** and PBA to BSA and/or OVA. The conjugations of haptens **1**−**5** with BSA or OVA were described previously (*33*).

Hapten Conjugates 7−BSA and −OVA. A 100 μL aliquot of butyl nitrite solution (123 μL/2 mL of DMSO) was added dropwise to a stirred solution of 9.4 mg (0.025 mmol) of hapten **7** in 1.05 mL of 37.5 mM H_2SO_4 in DMSO at 16 °C. Stirring was continued for 10 min, and the solution was divided in two equal aliquots. One aliquot was added to a solution of BSA and the other to a solution of OVA. Each protein solution consisted of 20 mg of protein dissolved in 5 mL of borate buffer (0.1 M, pH 9.4). The stirring was continued overnight at 4 °C. The solutions became yellow in color. The reaction mixtures were purified using a dextran-desalting column and phosphate-buffered saline (PBS, pH 7.5) as the eluent. The fractions of purified conjugates were easily detected because of their orange color. These fractions were pooled and stored in aliquots at −80, −20, and 4 °C.

Hapten Conjugates 5−Fetuin and 7−Fetuin. To a stirred solution of 24 mg (0.004 mmol) of fetuin in 4 mL of PBS was added 400 μL of aqueous $NaIO_4$ (100 mM). Stirring was continued for 20 min at room temperature. The solution was then dialyzed overnight at 4 °C against 1 mM sodium acetate buffer (pH 4.4). The dialyzed solution was transferred and aliquoted into two vials. To each vial was added 30 μL of 200 mM carbonate buffer (pH 9.7), followed by 18 mg (0.05 mmol) of each hapten (**5** or **7**), each in 1 mL of ethanol. The mixtures were stirred for 3 h at room temperature (after 1 h, 1 mL of THF was added to each to prevent precipitation of the haptens). Finally, 100 μL of freshly prepared $NaBH_4$ solution (4 mg/mL of water) was added to each vial, and the solutions were incubated overnight at 4 °C. The conjugates were purified and stored as described above.

Hapten Conjugates 8−BSA and 9−BSA. Compound **8** and **9** were coupled to BSA by the *N*-hydroxysuccinimide ester method of Langone and Van Vunakis (*35*). The acid hapten (0.015 mmol) was dissolved in 150 μL of dry DMF with equimolar NHS and 0.020 mmol of 1,3-dicyclohexylcarbodiimide. After the solution had been stirred for 3.5 h at room temperature, the precipitated dicyclohexylurea was removed by centrifugation, and the supernatant was added to 20 mg of BSA solution in 2.5 mL of distilled water and 0.5 mL of DMF. The reaction mixtures were stirred gently at 4 °C overnight. The solution was then dialyzed exhaustively against PBS and stored at −20 °C until it was used.

Conjugation of PBA−BSA. PBA (0.025 mmol) was dissolved in 2 mL of dry DMF, and then 6 mg (0.05 mmol) of NHS and 5.8 mg (0.03 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were added. The reaction mixture was stirred overnight at room temperature. BSA (50 mg) was dissolved in 10 mL of PBS and 2 mL of carbonate buffer (pH 9). The activated hapten was added dropwise to the protein solution. The mixture was stirred for 30 min at room temperature, and for 7 h at 4 °C. The solution was then dialyzed against PBS over the course of 72 h at 4 °C and stored at −20 °C.

Immunization. Immunizations were performed following the protocol reported previously (*36*). Three female New Zealand white rabbits were immunized intradermally with each immunogen (**5**− and **7**−fetuin). After the initial immunization with 100 μg of the immunogen in PBS, emulsified in Freund's complete adjuvant (1:1 v/v), further injections using Freund's incomplete adjuvant were given. Booster injections followed in 3-week intervals. The rabbits were bled 10 days after each immunization. Among the three rabbits immunized with **7**−fetuin, two rabbits (numbers 18 and 20) died prior to the final bleed. After coagulation of the blood, the serum was isolated after centrifugation for 10 min at 4 °C. Sera were collected prior to immunization to provide a control serum that shows no related humoral immune response. Antisera were screened for their specific antibody titer. Final bleeds were used for ELISA development.

Enzyme-Linked Immunosorbent Assay (ELISA). BSA and OVA conjugates were used as coating antigens diluted with a coating buffer [50 mM sodium carbonate/bicarbonate buffer (pH 9.6)]. Microtiter plates were usually coated overnight at 4 °C with 100 μL of the hapten−protein conjugate per well. After the plates had been washed with PBST {phosphate-buffered saline with Tween 20 [8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, and 0.05% Tween (v/v)]}, the surface of the wells was blocked with 200 μL of a BSA solution (0.5% in PBS) by incubating for 30 min at room temperature. After another washing step, 100 μL of PBS-diluted antiserum per well (for determination of antibody titer) or 50 μL of PBS-diluted antiserum per well and 50 μL of standard solution per well were dispensed into the wells and incubated for 60 min at room temperature. Following another washing step (four times), 100 μL of GAR−HRP per well (diluted 1:3000 in PBS) was added to each well, and the wells were incubated for 60 min at room temperature. The plate was washed four times, and 100 μL of substrate solution [3.3 μL of 30% H_2O_2 and 400 μL of 0.6% TMB in DMSO per 25 mL of acetate buffer (pH 5.5)] per well was pipetted into each well. Ten to fifteen minutes later, the color development was stopped by adding 50 μL of 2 M H_2SO_4 per well. The plates were then read in a dual-wavelength mode, subtracting the absorbance at 650 nm from the absorbance at 450 nm. All experiments were conducted using three or four well replicates. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, and they were fitted to the four-parameter logistic equation $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at zero analyte concentration, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% inhibition (I_{50}), and D is the minimum absorbance at infinite concentration.

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

Cross Reactivity. The optimized assays were submitted to cross-reactivity studies by using the standard solution of the analytes, other structurally related compounds (see Table 4), and 20 free amino acids. The cross reactivity (CR) was obtained from the metabolite standard and the related compound I_{50} values from the same plate, where % CR = (I_{50} of the metabolite/I_{50} of the tested compound) × 100.

Solvent Effects. Standard curves of analytes **8** and **9** were prepared in PBS buffer containing 0, 10, 20, 40, 60, and 80% (v/v) methanol to determine the effects of solvent.

Matrix Effects. (a) pH. Preparing analytes in phosphate buffers at pH 4, 6, and 8 tested the effect of pH. All other assay conditions were as described above.

(b) Ionic Strength. The effects of ionic strength on the quantitation of **8** and **9** were studied by preparing analyte standard solutions in 1×, 3×, 5×, and 8× PBS all at pH 7.5. Other assay conditions were the same as described above.

(c) Urine. The effects of the urine matrix on the quantitation of **8** and **9** were evaluated by preparing analyte standard solutions in a buffer with different concentrations (0, 1, 2, 4, and 10%) of urine. Four urine samples tested in this study were from healthy individuals without known exposure to pyrethroids.

Solid-Phase Extraction (SPE). The SPE method used in this study was modified from a previously reported method for the extraction of atrazinemercapturic acid from human urine using a C18 column (*32*). The C18 column (10 cm^3/500 mg; part no. 1211-3027, Varian Sample Preparation Products, Harbor City, CA) was preconditioned with 3 mL of methanol, deionized water, and 0.1 M phosphate buffer (pH 2.2). The urine samples (10 mL) were mixed with equal volumes of 0.1 M phosphate buffer (pH 2.2) and loaded on the column, eluting with 2−4 mL/min flows. Columns were then washed with 0.1 M phosphate buffer (pH 2.2), deionized water, and deionized water containing 20% methanol. After being dried under high vacuum for 15 min, the column was eluted with 3 mL of 100% methanol. The volume was adjusted to 4 mL with methanol, diluted 1:50 with 20% methanolic buffer, and analyzed with an ELISA.

Results and Discussion

Hapten Design and Synthesis. No study has reported which conjugate is predominant for pyrethroid metabolites in human urine. In mammals, the major "detoxication" reaction for carboxylic acid-containing xenobiotics is conjugation, with either an amino acid for forming a peptide or glucuronic acid for forming a glycoside (*22*, *23*). In general, larger and more complicated cyclic acids tend to be excreted as glycosides, but simpler acids such as benzoic and arylacetic acids are

Table 1. Summary of Titration Tests[a]

Ab/immunogen	**1**−BSA	**2**−BSA	**3**−BSA	**3**−OVA	**4**−BSA	**4**−OVA	**5**−BSA	**5**−OVA	**7**−BSA	**7**−OVA	**8**−BSA	**9**−BSA	PBA−BSA
15/**5**−fetuin	+[b]	+	+++	++	+++	+	++++[c]	++++[c]	−	−	++	−	−
16/**5**−fetuin	+	+	++	+	++	+	+++[c]	+++[c]	−	−	++	−	−
17/**5**−fetuin	+	+	+++	+++	+++	++	++++[c]	++++[c]	−	−	++++	−	−
19/**7**−fetuin	−	−	−	−	−	−	−	−	++++[c]	++++[c]	−	+++	+++

[a] The data shown are at an antibody dilution of 1:16000 and a coating antigen concentration of 1 mg/L. [b] Legend: −, <0.25; +, 0.25−0.50; ++, 0.50−0.75; +++, 0.75−1.00; and ++++, >1.00. Hapten structures of **1**−**5** are given in Figure 2 and of **7**−**9** in Scheme 1. [c] Homologous systems.

more commonly eliminated in the form of amino acid conjugates (*22*, *23*). Therefore, phenoxybenzoic acid, a primary breakdown metabolite of pyrethroids, and fenvalerate acid may predominantly undergo amino acid conjugation in humans. Glucuronidation is also an alternative conjugation pathway for these two acids; however, the possible internal transesterification makes these conjugates equilibrium mixtures, which are difficult to separate and detect. In contrast, the glycine conjugates are more stable and measurable. In this study, glycine conjugates of PBA and sFA were chosen as target analytes.

To generate a specific antibody for an analyte, it is important to alter the structure of the target compound as little as possible when it is modified for coupling to a carrier protein. In this respect, it is prudent to attach the handle as distally as possible from the partial structure that best defines the target. This was accomplished in the previous study for developing an esfenvalerate immunoassay by attachment of the handle at the 4-position of the terminal phenyl group of fenvalerate (haptens **1** and **2**). Similarly, in this study, hapten handles for glycine conjugates were attached to the amino acid, leaving the target acid unmodified (in haptens **5** and **7**). Syntheses of haptens were carried out using the chiral isomers where appropriate to facilitate purification as well as to make the chiral hapten corresponding to the major (active) isomer in esfenvalerate insecticide. 4-Nitro-L-phenylalanine having the same chirality as the natural L-phenylalanine was used as the intermediate in an effort to minimize handle recognition. The synthesis route for hapten **7** is summarized in Scheme 1. The acid chloride of 3-phenoxybenzoic acid was reacted through a modified Schotten-Baumann reaction with 4-nitro-L-phenylalanine to give the *N*-acylated nitro intermediate **6**. Subsequent catalytic reduction of the nitro group to the amine gave the corresponding amino hapten **7**.

The syntheses of the target metabolites **8** and **9** are summarized in Scheme 2. These were also prepared via Schotten-Baumann reactions of the respective acid chlorides with glycine and isolated by chromatography and crystallization. The structures of these compounds as well as all of the haptens were confirmed by NMR and mass spectroscopy.

Hapten Conjugation. Fetuin was chosen as a carrier protein for haptens **5** and **7**. For the conjugation of the haptens to fetuin, which is a glycoprotein, the $NaIO_4$ method was used. As discussed by Wengatz et al. (*37*), the following advantages were anticipated: (1) the increased carbohydrate content of the carrier increases the antigenicity of the immunogen; (2) the carbohydrate moiety allows the immunogen to be fairly hydrophilic despite loading with a lipophilic hapten; and (3) bovine fetuin is commercially available. The diazotization method was used in this study to prepare coating antigens. This conjugation method has the advantage of producing colored protein conjugates. The color can be seen as an indicator of a successful coupling reaction. The colored products are also easily detected when purified by gel chromatography.

Table 2. Selected Competitive ELISA Data

immunogen	analyte	antiserum	coating antigen	I_{50} (μg/L)
5−fetuin	sFA-glycine (**8**)	Ab 15	**3**−BSA	0.74[a]
			4−BSA	1.2
			5−BSA	8.2
		Ab 16	**3**−BSA	0.49
			4−BSA	0.49
			5−BSA	10
			8−BSA	2.6
		Ab 17	**3**−BSA	0.24
			4−BSA	0.34
			5−BSA	2.1
			8−BSA	1.5
7−fetuin	PBA-glycine (**9**)	Ab 19	**7**−BSA	2.5
			7−OVA	3.6
			9−BSA	1.2
			PBA−BSA	0.36

[a] Each set of data represents the average of four experiments.

Screening of the Sera and Assay Optimization. A checkboard titration system was used for screening of antibody and antigen combinations (*38*). The antisera of four rabbits were tested against 13 coating antigens, and the data are summarized in Table 1. In general, all antisera exhibited higher titers in homologous systems than in heterologous systems. In a homologous system, the fact that the hapten in the coating antigen and the hapten in the immunogen are identical is the primary reason for a higher titer. Antibodies raised against **5**−fetuin only recognized coating antigens containing the FA portion, and no titer was measured with PB-containing antigens. In contrast, antibodies raised against **7**−fetuin had a very high titer with PB-containing coating antigen **7**−BSA or −OVA, **9**−BSA, and PBA−BSA, and no recognition with any other antigens that were tested. The combinations of antibody and/or coating antigen with higher titers (OD > 0.5 in Table 1) were further screened by competitive assay. Since no difference (except **4**−BSA or −OVA) was observed in antibody titers between coating antigens with different carrier proteins (BSA or OVA), only BSA conjugates of **3**−**5** were used for further screening with Ab 15−17 against compound **8**. **7**−BSA, **9**−BSA, and PBA−BSA were tested for inhibition experiments with Ab 19 against compound **9**.

All combinations that were tested exhibited a very low I_{50} (<10 μg/L) against their target analytes (Table 2), which ranged from 10.0 to 0.30 μg/L. The homologous systems (Ab 15−17 vs cAg **5**−BSA; Ab 19 vs **7**−BSA) had higher I_{50}s than the heterologous systems. For example, with Ab 16, the I_{50} for analyte **8** in a homologous system is about 20 times higher than that in heterologous systems. These results are consistent with studies of

Table 3. Effects of Solvent (Methanol)[a]

analyte	Ab/cAg	% MeOH[b]	ABS_{max} (*A*)	slope (*B*)	I_{50} (*C*) (μg/L)	ABS_{min} (*D*)
sFA-glycine (**8**)	Ab 17/**3**−BSA	0	0.51	1.5	0.28 ± 0.03[c]	0.02
		10	0.52	1.1	0.18 ± 0.02	0.01
		20	0.55	1.2	0.31 ± 0.04	0.03
		40	0.64	1.1	0.99 ± 0.12	0.03
		60	0.66	0.94	4.9 ± 0.26	0.01
		80	0.67	0.95	22 ± 1.8	0.03
PBA-glycine (**9**)	Ab 19/PBA−BSA	0	0.55	0.80	0.45 ± 0.08	0.01
		10	0.60	0.82	0.30 ± 0.02	0.01
		20	0.65	0.80	0.25 ± 0.03	0.02
		40	0.72	0.77	0.33 ± 0.03	0.03
		60	0.81	0.71	1.0 ± 0.07	0.02
		80	0.77	0.55	4.2 ± 0.18	0.04

[a] ELISA conditions: 0.5 μg/mL coating antigen **3**−BSA, 1:16000 antiserum 17, 0.5 μg/mL coating PBA−BSA, 1:10000 Ab 19, and 1:3000 goat anti-rabbit IgG−HRP. [b] Concentration of MeOH in standard solution (PBS−MeOH). [c] Mean value ± SD. Each set of data represents the average of three experiments.

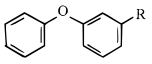
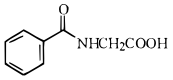
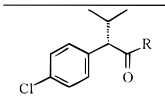
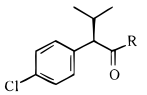
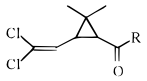
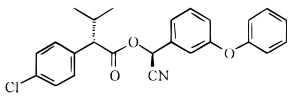
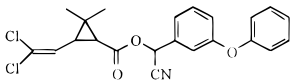
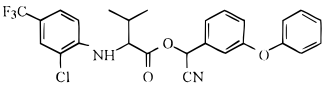
ELISAs for the triazines (*39*) and arylureas (*40*). Recently, Goodrow and Hammock (*41*) have reported the advantages of using heterologous haptens for immunogen and coating/tracer haptens and their uses for selective and sensitive assay design and development. In this study, only Ab 17/cAg **3**−BSA and Ab 19/cAg PBA−BSA were used for further assay development to detect **8** and **9**, respectively.

Solvent Effects. Methanol was evaluated for solvent effects in these two assays. The results (Table 3) show that methanol apparently influenced assay sensitivity and absorbance. The maximum absorbance (without analyte) for both assays was enhanced with increasing methanol concentrations. However, the I_{50} was increased when the solvent concentration exceeded 40% (for **9**) and 20% (for **8**) with decreasing slope values. Selection of the optimum MeOH concentration was based on the I_{50} values, the slope, and the ratios of maximum and minimum absorbances for standard curves (*A*/*D*). Generally, a lower I_{50}, at a higher MeOH concentration, and a high slope are desirable. In the study presented here, 20% MeOH was chosen in both assays for the analyte dilution and the standard solution preparation.

Cross Reactivity. Esfenvalerate, cypermethrin, fluvalinate, esfenvalerate metabolites, other structurally related compounds, and 20 free amino acids were tested for cross reactivities (Table 4). Both antibodies 17 and 19 are highly specific for their target analytes **8** and **9**, respectively. In all cases, the interference of the parent compound, other metabolites, and other tested compounds was negligible. Although PBA, PB alcohol, phenoxybenzoate, and three other tested pyrethroids contain the PB group, which is present in the immunogen hapten, they did not interfere in the assay for hapten **9** (CR < 0.5%). However, this antibody (Ab 19) binds to PBA−BSA very well (high titer in Table 1), indicating the important contribution of the handle (NH) recognition in the antibody specificity. The amide group, generated during coupling, in the PBA−BSA conjugate makes this antigen hapten structurally more like the target conjugates. Because free PBA and sFA are possible metabolites in urine (*18*, *21*), it is particularly significant that assays for both **8** and **9** have no cross reactivity with PBA and FA. This makes these assays very useful for selective detection of esfenvalerate metabolites.

Matrix Effects. Since these assays were intended to analyze urine samples, it was necessary to study the influence of pH and ionic strength in this matrix. Both assays are very sensitive to higher concentrations of salt

Table 4. Cross Reactivities of Esfenvalerate Metabolites and Other Related Compounds[a]

Structure	R	Cross-reactivity (%) 9	8
	$C(O)NHCH_2COOH$	100	0.01
	COOH	0.02	<0.01
	CH_2OH	0.01	<0.01
	$COOCH_3$	0.03	<0.01
		<0.01	<0.01
	$NHCH_2COOH$	<0.01	100
	OH	<0.01	0.08
	OCH_3	<0.01	0.30
	$NHCH_2COOH$	<0.01	2.80
	OH	<0.01	<0.01
	OCH_3	<0.01	0.01
 (esfenvalerate)		0.04	0.02
 (cypermethrin)		<0.01	<0.01
 (fluvalinate)		0.07	0.02

[a] Cross reactivity was calculated as (I_{50} of esfenvalerate/I_{50} of analyte) × 100%. Each set of data represents the average of three experiments.

in solution (Figures 3B and 4B). At an ionic strength of 3× PBS (0.45 M), the binding between antibody and antigen was suppressed approximately 50% in the assay of compound **9**, and almost completely inhibited for the assay of compound **8**. These results are consistent with the previous reported observations for atrazinemercapturic acid, which was assumed to result in disrupting Ab−Ag interaction (*32*).

These two assays also showed similar tolerance to the pH of the solutions (Figures 3A and 4A). Compared with PBS buffer (0.15 M, pH 7.5), no influences were observed at pH 6−8, indicating that a slight difference in the pH of sample or buffer (in this range) would not affect the

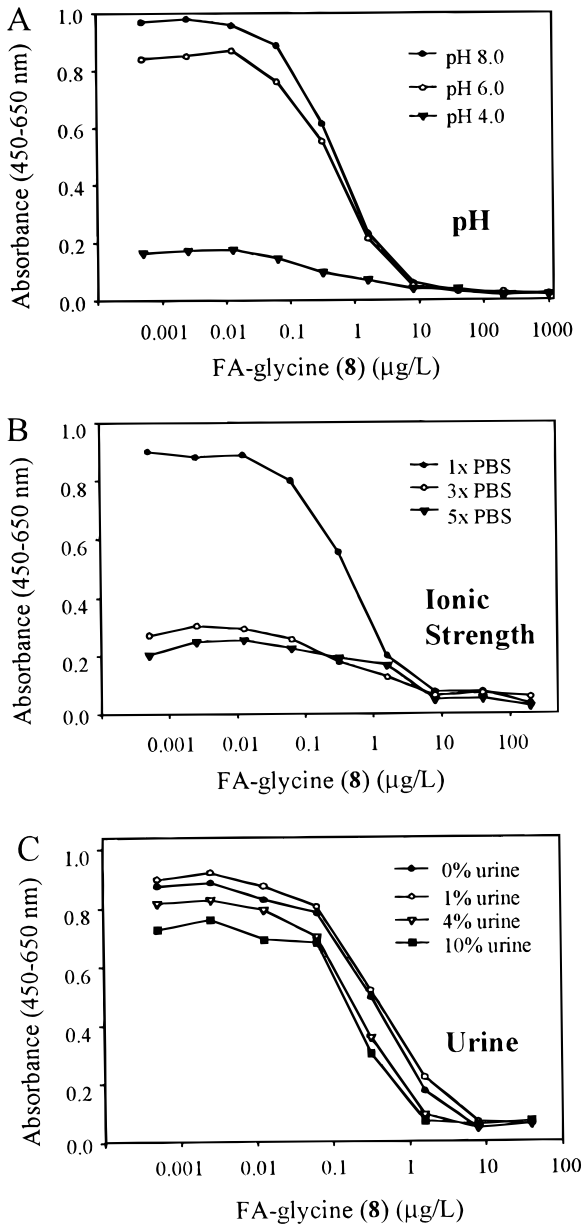


Figure 3. ELISA competition curves of FA-glycine (**8**) prepared with various (A) pHs, (B) ionic strengths, and (C) % urine. Reagent concentrations were as follows: coating antigen (**3**−BSA), 1:5000; antibody (Ab 17), 1:10000 (final concentration in wells); and goat anti-rabbit IgG−HRP, 1:3000.

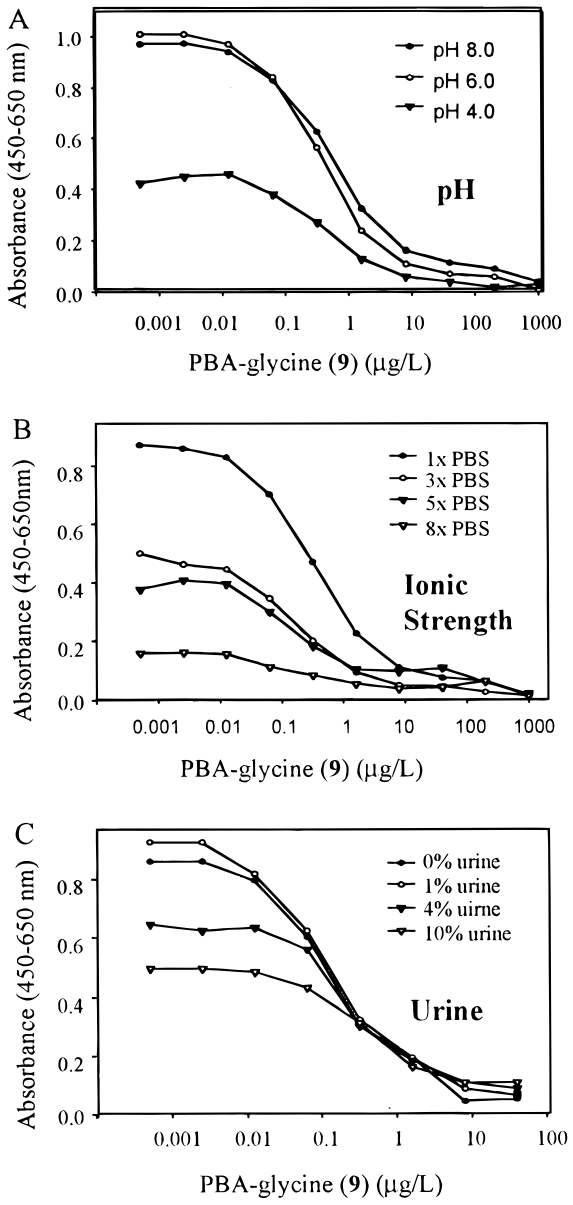


Figure 4. ELISA competition curves of PBA-glycine (**9**) prepared with various (A) pHs, (B) ionic strengths, and (C) % urine. Reagent concentrations were as follows: coating antigen (PBA−BSA), 1:10000; antibody (Ab 19), 1:20000 (final concentration in wells); and goat anti-rabbit IgG−HRP, 1:3000.

accuracy in the quantitation of **8** and **9**. However, both assays were severely suppressed at the more acidic pH of 4.0. Therefore, these assays required the urine samples to be adjusted by dilution or by a desalting method prior to ELISA detection.

The effects of the urine matrices were evaluated for both assays. In tests with four different urine samples, the assay for **8** could tolerate 4% urine, while the assay for **9** required 100-fold dilution prior to the ELISA to reduce the effects of urine matrix interference (Figures 3C and 4C). The results suggest that these assays may actually be inhibited by endogeneous urinary compounds. Therefore, dilution prior to ELISA would be necessary for both assays. When the variation of the different urine samples is considered, a 150-fold dilution of the urine before the assay was chosen for both assays. In recovery experiments for the direct dilution of urine samples, urine matrix had little effect on accurate quantitation for urines

Table 5. Spiked Recovery

spiked concentration (μg/L)	compound **9**		compound **8**	
	SPE	dilution	SPE	dilution
0	<0.2	0.62 ± 0.44	<0.2	0.84 ± 0.73
1.0	1.09 ± 0.22	1.80 ± 0.82	1.15 ± 0.26	1.60 ± 0.90
2.0	2.16 ± 0.32	2.85 ± 0.87	2.06 ± 0.40	2.78 ± 1.0
5.0	5.31 ± 0.42	5.88 ± 1.0	4.89 ± 0.29	5.40 ± 0.67
10	10.5 ± 0.10	11.7 ± 0.94	9.54 ± 0.30	11.2 ± 0.89
50	49.0 ± 1.5	52.0 ± 1.5	48.7 ± 1.0	54.0 ± 2.8

spiked with ≥5.0 μg/L **8** (18.5 nmol/L) and ≥5.0 μg/L **9** (18.3 nmol/L) (Table 5). The recovery rates for **8** and **9** at 5.0 μg/L are 108 + 12.3% and 117 + 21.0%, respectively. However, urine interfered significantly with the quantitation of unspiked samples and urines spiked with **8** or **9** at a concentration of <5.0 μg/L (Table 5). According to the recommended LOQ determination guideline (*42*), the approximate limit of quantitation is 5.0 μg/L for both **8** and **9**. This is consistent with the estimated concentra-

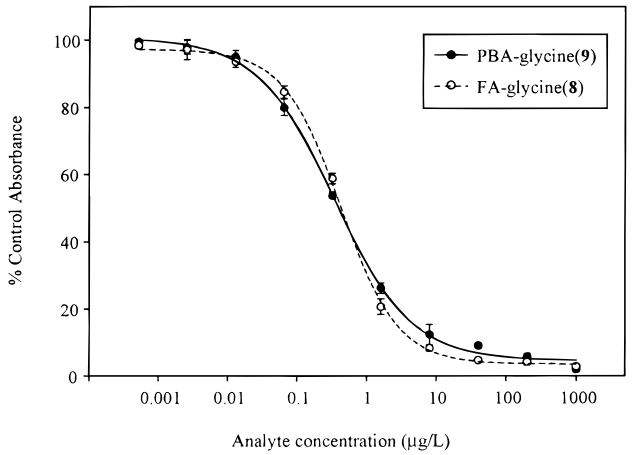


Figure 5. ELISA inhibition curves for PBA-glycine (**9**) and FA-glycine (**8**). Reagent concentrations were as follows: the assay of **8** using antiserum 17, 1:10000 (final dilution in wells); and coating antigen **3**−BSA, 1:5000; and the assay of **9** using antiserum 19, 1:20000 (final dilution in wells); and coating antigen PBA−BSA, 1:10000. Both standard curves represent the average of 24 curves.

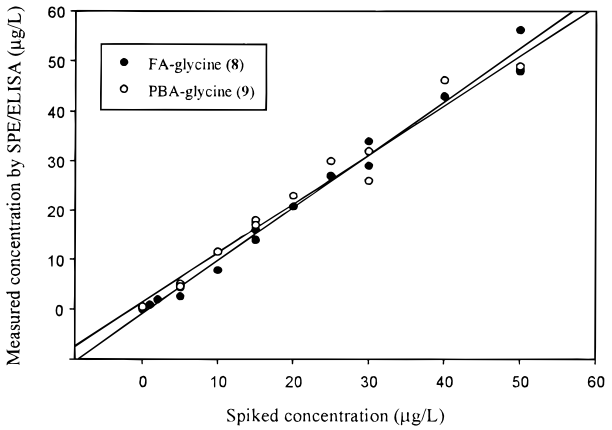


Figure 6. Relationship between analyte levels spiked into urine and measured by SPE/ELISA. Urine samples were from individuals with no known exposure to pyrethroids. Studies were carried out in a blind fashion. For sFA-glycine (**8**), $y = 1.06x - 1.48$, $r^2 = 0.99$, and $n = 16$. For PBA-glycine (**9**), $y = 0.99x + 0.77$, $r^2 = 0.97$, and $n = 15$.

tion that corresponded to the absorbance of the control (zero analyte) minus 3 times the standard deviation of control, when standard analytes were prepared in a buffer system with 0.67% unspiked urine.

Solid-Phase Extraction (SPE). A C18 column was used for urine sample extraction in this study. The SPE method was based on previously reported methods for isolating atrazinemercapturic acid from human urine (*32*). These assays are sensitive to acidic conditions; therefore, the final washing step was changed by using deionized water and 20% methanolic water (deionized). Good recovery and less interference were achieved for both assays using this washing step (Table 5). However, SPE under these conditions failed to eliminate all matrix effects, and dilution of the concentrated sample prior to the ELISA was required for accurate quantitation. According to the test of four urine samples, a 25-fold dilution could eliminate the interference of the urine matrix. When the variability of different urine samples was considered, a 50-fold dilution was used for both assays in this study. Similarly, the LOQ for the detection of **8** and **9** can be defined as 1.0 μg/L (3.70 nmol/L) (Table 5).

Assay Validation. The assay validation was performed in a blind fashion by direct dilution or SPE sample preparation of the urine samples, which were spiked with **8** or **9** ranging in concentration from 0 to 50 μg/L (Figure 5). The linear regression analysis of ELISA results showed a very good correlation in all four cases (Figure 6). The data from samples prepared by SPE had a slightly better correlation ($r^2 = 0.99$ and 0.97 for **8** and **9**, respectively) than those prepared by direct dilution ($r^2 = 0.89$ and 0.90 for **8** and **9**, respectively). All recoveries were >93% of the spiked values. The results demonstrate that these assays are suitable for the quantitative detection of esfenvalerate metabolite conjugates at trace levels in urine samples.

Both of the sample preparation methods had advantages and disadvantages. Direct dilution is easier and faster, while the SPE method has a lower LOQ for the detection of **8** and **9** in human urine. The dilution method would be more amenable than SPE to the rapid analysis of large numbers of urine samples, and the SPE method would be more accurate and useful when a lower LOQ is needed. In conclusion, a highly sensitive, specific, and reproducible ELISA has been developed and characterized for the detection of the fenvalerate metabolites **8** and **9**. The linear ranges of the extent of inhibition of **8** and **9** in buffer are approximately 0.03−60 and 0.04−50 μg/L, respectively, and the I_{50} values for **8** and **9** are 0.40 (1.47 nmol/L) and 0.42 μg/L (1.56 nmol/L), respectively. Organic solvents, temperature, incubation time, and human urine had strong matrix effects on the optimized immunoassays. The ELISAs were successfully applied to the quantitative detection of trace amounts of **8** and **9** in human urine by using either direct dilution or the SPE method. Since phenoxybenzyl (PB) is a common group in conventional pyrethroids, the assay of compound **9** is class-specific for PB-containing pyrethroids, which makes this assay more useful and significant as a biomarker of exposure to pyrethroids. Finally, further studies of human urine from individuals who have been exposed to esfenvalerate or other pyrethroids are necessary for toxicological and analytical confirmation.

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References

(1) Leahey, J. P. (1985) *The Pyrethroid Insecticides*, p 14, Taylor & Francis, London.

(2) Casida, J. E., and Ruzo, L. O. (1980) Metabolic chemistry of pyrethroid insecticides. *Pestic. Sci.* **11**, 257−269.

(3) Miyamoto, J., Beynon, K. I., Roberts, T. R., Hemingway, R. J., and Swaine, H. (1981) The chemistry, metabolism and residue analysis of synthetic pyrethroids. *Pure Appl. Chem.* **53**, 1967−2022.

(4) Aldridge, W. N. (1990) An assessment of the toxicological properties of pyrethroids and their neurotoxicity. *Toxicology* **21**, 89−104.

(5) Narahashi, T. (1992) Nerve membranes as targets of insecticides. *Trends Pharmacol. Sci. 13*, 236−241.

(6) Miyamoto, J., Kaneko, H., Tsuji, R., and Okuno, Y. (1995) Pyrethroids nerve poisons-how their risks to human health should be assessed. *Toxicol. Lett.* **82**−**83**, 933−940.
(7) Class, T. J., and Kintrup, J. (1991) Pyrethroids as household insecticides-analysis, indoor exposure and persistence. *Fresenius J. Anal. Chem.* **340**, 446−453.
(8) He, F., Sun, J., Han, K., Wu, Y., and Yao, P. (1988) Effects of pyrethroid insecticides on subjects engaged in packaging pyrethroids. *Br. J. Ind. Med.* **45**, 548−551.
(9) He, F., Wang, S., Liu, L., Chen, S., Zhang, Z., and Sun, J. (1989) Clinical manifestations and diagnosis of acute pyrethroid poisoning. *Arch. Toxicol.* **63**, 54−58.
(10) Hallenbeck, W. H., and Cunningham-Burns, K. M. (1985) *Pesticides and Human Health*, p 118, Springer-Verlag, New York.
(11) Repetto, R. C. (1996) in *Pesticides and the immune system: the public health risks* (Repetto, R., and Baliga, S. S., Eds.) World Resources Institute, National Center for Food & Agricultural Policy, Washington, DC.
(12) Go, V., Garey, J., Wolff, M. S., and Pogo, G. T. (1999) Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. *Environ. Health Perspect.* **107**, 173−177.
(13) Adamis, Z., Antal, A., Fuezesi, I., Molnar, J., Nagy, L., and Susan, M. (1985) Occupational exposure to organophosphorous insecticides and synthetic pyrethroid. *Arch. Occup. Environ. Health* **56**, 299−305.
(14) Crawford, M. J., Croucher, A., and Hutson, D. H. (1981) The metabolism of the pyrethroid insecticide cypermethrin in rats: excreted metabolites. *Pestic. Sci.* **12**, 393−411.
(15) Casida, J. E. (1983) Novel aspects of metabolism of pyrethroids. In *Pesticide Chemistry: Human Welfare and the Environment* (Miyamoto, J., and Kearney, P. C., Eds.) Vol. 2, pp 187−192, Pergamon, New York.
(16) Mijamoto, J., Kaneko, H., Hutson, D. H., Esser, H. O., Gorbach, S., and Dorn, E. (1988) *Pesticide Metabolism: Extrapolation from Animals to Man*, p 35, Blackwell Scientific Publications, Oxford, U.K.
(17) Kuhn, K.-H., Leng, G., Bucholski, K. A., Dunemann, L., and Idel, H. (1996) Determination of pyrethroid metabolites in human urine by gas chromatography−mass spectrometry. *Chromatographia* **43**, 285−292.
(18) Ohkawa, H., Kaneko, H., Tsuji, H., and Miyamoto, J. (1979) Metabolism of fenvalerate (Sumicidin) in rats. *J. Pestic. Sci.* **4**, 143−155.
(19) Eadsforth, C. V., and Baldwin, M. K. (1983) Human dose excretion studies with the pyrethroid insecticide cypermethrin. *Xenobiotica* **13**, 67−72.
(20) Eadsforth, C. V., Bragt, P. C., and VanSittent, N. J. (1988) Human dose-excretion studies with pyrethroid insecticides cypermethrin and alphacypermethrin: relevance for biological monitoring. *Xenobiotica* **18**, 603−614.
(21) Isobe, N., Kaneko, H., Shiba, K., Saito, K., Ito, S., Kakuta, N., Saito, A., Yoshitake, A., and Miyamoto, J. (1990) Metabolism of esfenvalerate in rats and mice and effects of its isomers on metabolic fates of esfenvalerate. *J. Pestic. Sci.* **15**, 159−168.
(22) Killenberg, P. G., and Webster, L. T., Jr. (1980) Conjugation by peptide-bond formation. In *Enzymatic Basis of Detoxication* (Jakoby, W. B., Ed.) Vol. II, Academic Press, New York.
(23) Sipes, J. G., and Gandolfi, A. J. (1991) Biotransformation of toxicants. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Amdur, M. O., Doull, J., and Klaassen, C. D., Eds.) 4th ed., Pergamon, New York.
(24) Woollen, B. H., Marsh, J. R., Laird, W. J. D., and Lesser, J. E. (1992) The metabolism of cypermethrin in man: differences in urinary metabolite profiles following oral and dermal administration. *Xenobiotica* **22**, 983−991.
(25) Leng, G., Kuhn, K.-H., and Idel, H. (1997) Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: applications and limitations. *Sci. Total Environ.* **199**, 173−181.
(26) Leng, G., Leng, A., Kuhn, K.-H., Lewalter, J., and Pauluhn, J. (1997) Human dose-excretion studies with the pyrethroid insecticide cyfluthrin: urinary metabolite profile following inhalation. *Xenobiotica* **27**, 1273−1283.
(27) Angerer, J., and Ritter, A. (1997) Determination of metabolites of pyrethroids in human urine using solid-phase extraction and gas chromatography−mass spectrometry. *J. Chromatogr., B* **695**, 217−226.
(28) Harris, A. S., Lucas, A. D., Kramer, P. M., Marco, M.-P., Gee, S. J., and Hammock, B. D. (1995) The use of immunoassays for the detection of urinary biomarkers of exposure. In *Frontiers in Agrochemical Immunoanalysis* (Kurtz, D., Stanker, L., and Skerritt, J., Eds.) AOAC, Washington, DC.
(29) Hammock, B. D., Gee, S. J., Harrison, R. O., Jung, F., Goodrow, M. H., Li, Q. X., Lucas, A., Szekacs, A., and Sundaram, K. M. S. (1990) Immunochemical technology in environmental analysis: addressing critical problems. In *Immunochemical Methods for Environmental Analysis* (Van Emon, J., and Mumma, R., Eds.) pp 112−139, ACS Symposium Series 442, American Chemical Society, Washington, DC.
(30) Lucas, A. D., Jones, A. D., Goodrow, M. H., Saiz, S. G., Blewett, C., Seiber, J. N., and Hammock, B. D. (1993) Determination of atrazine metabolites in human urine: Development of a biomarker of exposure. *Chem. Res. Toxicol.* **6**, 107−115.
(31) Marco, M. P., Nasiri, M., Kurth, M. J., and Hammock, B. D. (1993) Enzyme-linked immunosorbent assay for the specific detection of the mercapturic acid metabolites of naphthalene. *Chem. Res. Toxicol.* **6**, 284−293.
(32) Jaeger, L. L., Jones, A. D., and Hammock, B. D. (1998) Development of an enzyme-linked immunosorbent assay for atrazine mercapturic acid in human urine. *Chem. Res. Toxicol.* **11**, 342−352.
(33) Shan, G.-M., Stoutamire, D. W., Wengatz, I., Gee, S. J., and Hammock, B. D. (1999) Development of an immunoassay for the pyrethroid insecticide esfenvalerate. *J. Agric. Food Chem.* **47**, 2145−2155.
(34) Tijssen, P. (1985) Outline of the strategies for enzyme immunoassays. In *Practice and Theory of Enzyme Immunoassays* (Burdon, R. H., and van Knippenberg, P. H., Eds.) pp 9−20, Elsevier Science Publishers, Amsterdam, The Netherlands.
(35) Langone, J. J., and Van Vunakis, H. (1975) Radioimmunoassay for dieldrin and aldrin. *Res. Commun. Chem. Pathol. Pharmacol.* **10**, 163−171.
(36) Gee, S. J., Miyamoto, T., Goodrow, M. H., Buster, D., and Hammock, B. D. (1988) Development of an enzyme-linked immunosorbent assay for the analysis of the thiocarbamate herbicide molinate. *J. Agric. Food Chem.* **36**, 863−870.
(37) Wengatz, I., Stoutmire, D. W., Gee, S. J., and Hammock, B. D. (1998) Development of an enzyme-linked immunosorbent assay for detection of the pyrethroid insecticide fenpropathrin. *J. Agric. Food Chem.* **46**, 2211−2221.
(38) Gee, S. J., Hammock, B. D., and Van Emon, J. M. (1994) *A User's Guide to Environmental Immunochemical Analysis*, EPA/540/R-94/509, Office of Research and Development, Environmental Monitoring Systems Laboratory, United States Environmental Protection Agency, Las Vegas, NV.
(39) Harrison, R. O., Goodrow, M. H., and Hammock, B. D. (1991) Competitive inhibition ELISA for the s-triazine herbicides: Assay optimization and antibody characterization. *J. Agric. Food Chem.* **39**, 122−128.
(40) Schneider, P., Goodrow, M. H., Gee, S. J., and Hammock, B. D. (1994) A highly sensitive and rapid ELISA for the arylurea herbicides diuron, Monuron, and linuron. *J. Agric. Food Chem.* **42**, 301−309.
(41) Goodrow, M. H., and Hammock, B. D. (1998) Hapten design for compound-selective antibodies: ELISAs for environmentally deleterious small molecules. *Anal. Chim. Acta* **376**, 83−91.
(42) Brady, J. F. (1995) Interpretation of immunoassay data. In *Immunoanalysis of Agrochemicals, Emerging Technologies* (Nelson, J. O., Karu, A. E., and Wong, R. B., Eds.) pp 267−287, American Chemical Society, Washington, DC.

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