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High-Throughput Automated Luminescent Magnetic Particle-Based Immunoassay to Monitor Human Exposure to Pyrethroid Insecticides

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We have developed a sensitive, automated, competitive chemiluminescent immunoassay for the detection of 3-phenoxybenzoic acid (3-PBA), a metabolite common to many pyrethroid insecticides. The system uses a competitive hapten−protein conjugate that has been labeled with an acridinium ester as the chemiluminescent probe and secondary antibody-coated paramagnetic particles for the separation. After the immunoassay reagents and samples are combined for the competitive incubation step, a fully automated system is used to load the postincubation mixture into a delivery cuvette, facilitating the subsequent magnetic separation of the immunocomplex and the measurement of chemiluminescent signal for quantification. The immunoassay format described here supports the requirement for high throughput necessary for monitoring large numbers of samples in population-based studies. The optimized immunoassay was more sensitive than the conventional enzyme immunoassay in buffer (IC_{50} = 0.1 and 2 μg/L, respectively). The mixed-mode solid-phase extraction used for sample preparation to reduce possible urinary matrix effects allowed the accurate measurement of 3-PBA levels as low as 1 μg/L. The automated chemiluminescent immunoassay described here is sensitive, simple to use, and more rapid than the previously reported standard microplate assay.

High-throughput screening (HTS) technologies provide speed and efficiency, allowing many measurements to be made in a short period of time, thus facilitating the acquisition of large quantities of high-quality data. Automation is a critical contributor to HTS methodologies. Specialized robotics are often used for much of the assay process, from loading samples through final data analysis. An HTS robot can usually prepare and analyze many HTS plates simultaneously, further speeding the data collection process. HTS robots that can test up to 100 000 compounds per day currently exist.[1] Zweigenbaum and Henion[2] demonstrated that an HTS-enabled tandem LC/MS (liquid chromatography-mass spectrometry) instrument equipped with an autosampler, automated sample preparation, and data analysis software could significantly reduce the time and labor required for chromatographic separation and detection of multiple analytes in human plasma samples.

Recently, immunoassay automation has emerged as an innovative area for research and development in the clinical diagnostic industry. In this case, automation can reduce labor requirements, costs, and turnaround times. Automation has also been demonstrated to result in improved assay performance based on individual improvements in assay precision, sensitivity, dynamic range, as well as the elimination of sample handling/processing and data-reduction errors resulting from manual operations.[3] Multiplexed fluorescence-based immunoassay technology has recently been introduced as an automatic high-throughput format for multianalyte screening. It has been shown that fluorescent flow-cytometric assays combined with the use of differently sized or fluorescently labeled polystyrene microbeads can be used to perform specific and quantitative immunoassays for multiple biomarker analytes,[4] e.g., cytokines as indicators of inflammation,[5−8] and environmental analytes, e.g., glyphosate, atrazine, and the mercapturate of metolachlor[9] in urine of exposed farm workers. Although, according to one manufacturer, it is theoretically possible to expand this technique to up to 100 analytes, it must be considered that cross-reacting analytes can complicate the interpretation of results and the selection of multiple analytes will be limited by the sample preparation methods and assay conditions required for each analyte.

Unlike the multiplexed bead immunoassays, automated chemiluminescent (CL) immunoassay is used to measure a single analyte per sample. Nevertheless, CL-based immunoassays are

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emerging as an attractive option. CL immunoassays do not require an enzymatic substrate, an inhibitor to stop the reaction, or an excitation source for a fluorescent dye. Sensitive assay methods can be developed and optimized due to the high quantum yield and the rate for the CL reaction and the efficiency of the light detector as an alternative to the colorimetric detection of enzyme-conjugated immunoassays (ELISA).[10,11] In place of the chromogenic substrate used in the conventional ELISA, examples of CL signal include luminol oxidation by hydrogen peroxide (H_2O_2) and the enzyme horseradish peroxidase (HRP)[12] and the enzyme-independent oxidation of acridinium ester in dilute alkaline hydrogen peroxide.[13] The reaction rate of CL-based signal generation is fast compared to colorimetric ELISA methods with a signal lifetime of 1−2 min.

Automated CL immunoassays, performed by the ACS:180 analyzer used for the study reported here have been performed for diagnostic clinical chemistry applications such as the assays related to anemia, cardiac, oncology, reproductive, thyroid, and therapeutic drug monitoring.[14] The ACS:180 analyzer automatically performs up to 180 analyses per hour using paramagnetic particles for the automatic separation of the immunocomplex from the assay supernatant in a heterogeneous assay format. Direct chemiluminescence from an acridinium ester-labeled probe is used for high assay sensitivity. The ACS system is attractive for the rapid measurement of single analytes of interest and has been used for the sulfonylurea herbicide triasulfuron in soil samples (IC_{50} = 0.1 μg/L).[15]

Pyrethroid insecticides are widely used in agricultural, industrial, and residential applications to control insects. Since the organophosphate insecticides chlorpyrifos and diazinon have been phased out for residential applications due to suspected human toxicities, the use of pyrethroids has increased as a safer alternative class of insecticides. Although little is presently known about the hazardous effects of human exposure, animal studies have demonstrated that pyrethroid exposure may affect neurological development,[16,17] induce a cancer,[18] suppress the immune system,[19] and disrupt the endocrine systems.[20−22]

Recently, the Center for Disease Control and Prevention[23] reported that 3.3 μg/L was the 95th percentile urine concentration of 3-phenoxybenzoic acid (3-PBA), a general urinary metabolite and biomarker of exposure to some pyrethroid insecticides, found in much of the U.S. population. This value is similar to the 2 μg/L reference value set by the German Federal Environmental Agency,[24] indicating wide-ranging exposure. 3-PBA levels as low as 0.1 μg/L have been measured together with urinary biomarkers that indicate specific pyrethroid exposure (*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), 4-fluoro-3-phenoxybenzoic acid (F-PBA), and *cis*-3-(2,2-dibromovinyl-2,2-dimethylcyclopropane carboxylic acid (DBCA)) using an improved high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method.[25] Instrumental methods such as gas chromatography (GC) or HPLC combined with mass spectrometry (MS) are currently the methods of choice for large scale biomonitoring studies that screen for multiple analytes[25,26] and environmental monitoring.[27] Although these chromatographic MS techniques are well documented and give accurate results, they are prohibitive because of their requirements for personnel and budget necessary for the operation and maintenance of complex instrumentation, expensive isotopic standards, and complex sample preparation methods including extraction, cleanup, and possibly chemoselective derivatization.

As an alternative, immunoassays have been proven to be rapid, sensitive, relatively simple, and cost-effective analytical tools for routine monitoring applications.[28] Immunoassays can be formatted to use only a small volume of sample and prepared with relatively simple methods, as compared to those needed for subsequent instrumental analyses. Immunoassays are readily adaptable to high-throughput methods using the 96- or 384-microwell plates or cuvette-based autoanalyzer systems.[3]

The goal of this study was to adapt an immunoassay for a urinary biomarker of pesticide exposure to the well-established ACS:180 analyzer system currently used to measure many indicators of clinical effect. Thus, a researcher could use a single platform technology to measure both markers of exposure and effect. Such a strategy could be useful in geographic locations where economics limits technology or technology infrastructure.

EXPERIMENTAL SECTION

Chemicals and Instruments. Horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin (OVA), *N*-hydroxysuccinimide (NHS), and 1,3-dicyclohexylcarbodiimide (DCC), 3-phenoxybenzoic acid (3-PBA), Tween 20, acetonitrile (ACN), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF),

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casein, and NaN_3 were purchased from Sigma-Aldrich (St. Louis, MO). Acridinium C2 NHS ester (4-(2-succinimidyl-oxycarbonyl-ethyl) phenyl-10-acridinium-9-carboxylate trifluoromethyl sulfonate) was obtained from Assay Designs, Inc. (Ann Arbor, MI). Trigger solutions no. 1 and no. 2 to generate chemiluminescent signal were obtained from Bayer/Siemens (Los Angeles, CA). Goat antirabbit IgG-coated paramagnetic particles were obtained from Qiagen (San Diego, CA). Econo-Pac 10DG gel filtration columns were obtained from Biorad (Hercules, CA). The C18 solid-phase extraction (SPE) columns (500 mg/10 mL) were obtained from Varian (Harbor City, CA), and the Strata Screen-A mixed-mode SPE columns (200 mg/3 mL) were obtained from Phenomenex (Torrance, CA). A vacuum manifold having 20 ports was used for the cleanup of urine samples prior to immunoassay. The automated CL immunoassay was run on the ACS:180 bench top immunoassay analyzer (Bayer, Emeryville, CA). For the cross-reactivity test, 4-hydroxy-3-phenoxybenzoic acid, 4-fluoro-3-phenoxybenzoic acid, a glycine conjugate of 3-phenoxybenzoic acid, and a glucuronide conjugate of 3-phenoxybenzyl alcohol were synthesized in this laboratory.

Immunoreagents and Buffers. The specific antibody and competing heterologous hapten for the target analyte (3-PBA) were previously described by Shan et al.[29] Briefly, the polyclonal antibody (Ab 294) was produced against a conjugate of 3-((2-oxoethoxy)ethoxy)phenoxybenzoic acid and thyroglobulin. Potassium phosphate casein buffer (14.04 g/L $K_2HPO_4 \cdot 3H_2O$, 5.24 g/L KH_2PO_4, 1 g/L NaN_3, and 5 g/L casein, pH 7.0), normal strength PBS buffer (1× PBS; 8 g/L NaCl, 0.2 g/L KH_2PO_4, 1.2 g/L Na_2HPO_4, and 0.2 g/L KCl, pH 7.5), PBST (1× PBS containing 0.05% (v/v) Tween 20, pH 7.5), PBSB (1× PBS containing 0.5% (w/v) BSA, pH 7.5), and 0.05 M borate buffer (19.1 g/L $Na_2B_4O_7 \cdot 10H_2O$, pH 8) were used for the immunoassay.

Preparation of Acridinium Ester-Labeled Hapten−Protein Conjugate. The preparation of the 3-PBA−protein−acridinium ester (3-PBA−protein−A^+) conjugate is illustrated in Figure 1. Briefly, 3-PBA (6.5 mg, 0.03 mmol) was dissolved in 0.4 mL of dry DMF with NHS (5.2 mg, 0.045 mmol) and DCC (9.2 mg, 0.045 mmol). After 5 h of stirring at room temperature, the precipitated dicyclohexylurea was removed through filtration, and the amount (20-fold molar excess over protein) of the resulting activated ester solution of 3-PBA was added slowly to a solution of either HRP, OVA, or BSA in 1 mL of 0.05M borate buffer (pH 8.5). The mixture was stirred gently at 4 °C for 24 h to complete the conjugation, followed by dialysis against normal strength phosphate-buffered saline (1× PBS, pH 7.5) which was changed twice a day for 3 days to remove unreacted small molecules.

The acridinium C2 NHS ester (A^+, 1.1 mg) was dissolved in 0.55 mL of dry DMF. A 120 μL aliquot (5-fold molar excess of the acridinium ester over protein) of the total ester solution was slowly added with vigorous stirring into the 3-PBA−protein solution that had been adjusted to pH 8 with drops of 1 N NaOH. The mixture was stirred at room temperature. After 20 min, 100 μL of 10% lysine in the PBS (pH 8) was added and stirred for 15 min at room temperature to quench unreacted label. The resulting mixture was then transferred to an Econo-Pac 10DG gel filtration column, which had been washed and equilibrated with the PBS buffer, 1 mL fractions were collected, and the chemiluminescence activity of each fraction was measured. Fractions collected between 4 and 6 mL elution volumes were found to contain 3-PBA−protein−A^+ conjugate and were combined with 3 mL of 50% glycerin in water, aliquoted, and stored at less than −70 °C until use.

Analysis of the Densities of Hapten and Acridinium Tracer Labeled on BSA. For the determination of the degree of 3-PBA conjugation to BSA and of the degree of acridinium tracer conjugated to 3-PBA−protein conjugate, 3-PBA−BSA conjugate and the 3-PBA−BSA−A^+ conjugate were dialyzed against distilled water for 24 h to remove salts, and then a powder of each was obtained by lyophilization. The density of hapten conjugated to protein and that of acridinium tracer conjugated to 3-PBA−BSA conjugate was determined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard BSA with that of the conjugates. MALDI spectra were obtained by mixing 0.5 μL of matrix (sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 5 mg/mL in 0.1% trifluoroacetic acid (TFA) in 65% ACN/water) and 0.5 μL of a solution of the conjugates (5 mg/mL in 5% formic acid) and spotting 0.6 μL to the MALDI target.

Automated Competitive Chemiluminescent Immunoassay for 3-PBA. The overall scheme for the 3-PBA assay formatted for the ACS:180 is illustrated in Figure 1. The assay was divided into the off-line competition step and, thereafter, to the on-line steps for immunocomplex separation and CL signal measurement. Briefly, 150 μL each of 3-PBA−protein−A^+ diluted in PBS containing 0.05% Tween 20 and polyclonal antibody diluted in the buffer was mixed in a 5 mL disposable plastic tube and preincubated for 30 min at room temperature. Equal volumes of 3-PBA standards or urine samples diluted in the buffer were added by pipet and incubated for at least 30 min at room temperature for the competition step. For the automatic, on-line steps, 200 μL aliquots of the mixture in each assay tube were added to each cuvette. After 7 min of incubation at 37 °C in the instrument, a 250 μL aliquot of goat antirabbit paramagnetic particles (GRPP) diluted 1:4 with buffer was added to each cuvette, and the resultant mixture was incubated for a further 5 min at 37 °C. The particle-adsorbed immunocomplexes in each cuvette were then automatically washed twice with 250 μL of deionized water by magnetic separation, and the CL signal was determined by adding a 250 μL aliquot of the trigger solution no. 1 to each cuvette with an equal volume of the trigger solution no. 2. The CL emission was then integrated over 5 s and expressed as photon counts. Finally, the signal of the buffer only (blank) was subtracted, and the data were plotted using a four-parameter logistic curve fitting algorithm.

Cross-Reactivity. The optimized assay was used to run cross-reactivity (CR) studies by using a standard solution of the permethrin metabolites and other structurally related compounds in MeOH stock solutions. The test compounds are listed in Table 1. The CR was calculated as a percentage with the following formula: (IC_{50} of the target analyte/IC_{50} of the tested compound) ×100%.

Urine Sample Preparation. *Cleanup with C18 Solid-Phase Extraction (C18 SPE).* Urine samples were kept at −20 °C without any treatment. After thawing, each sample was completely

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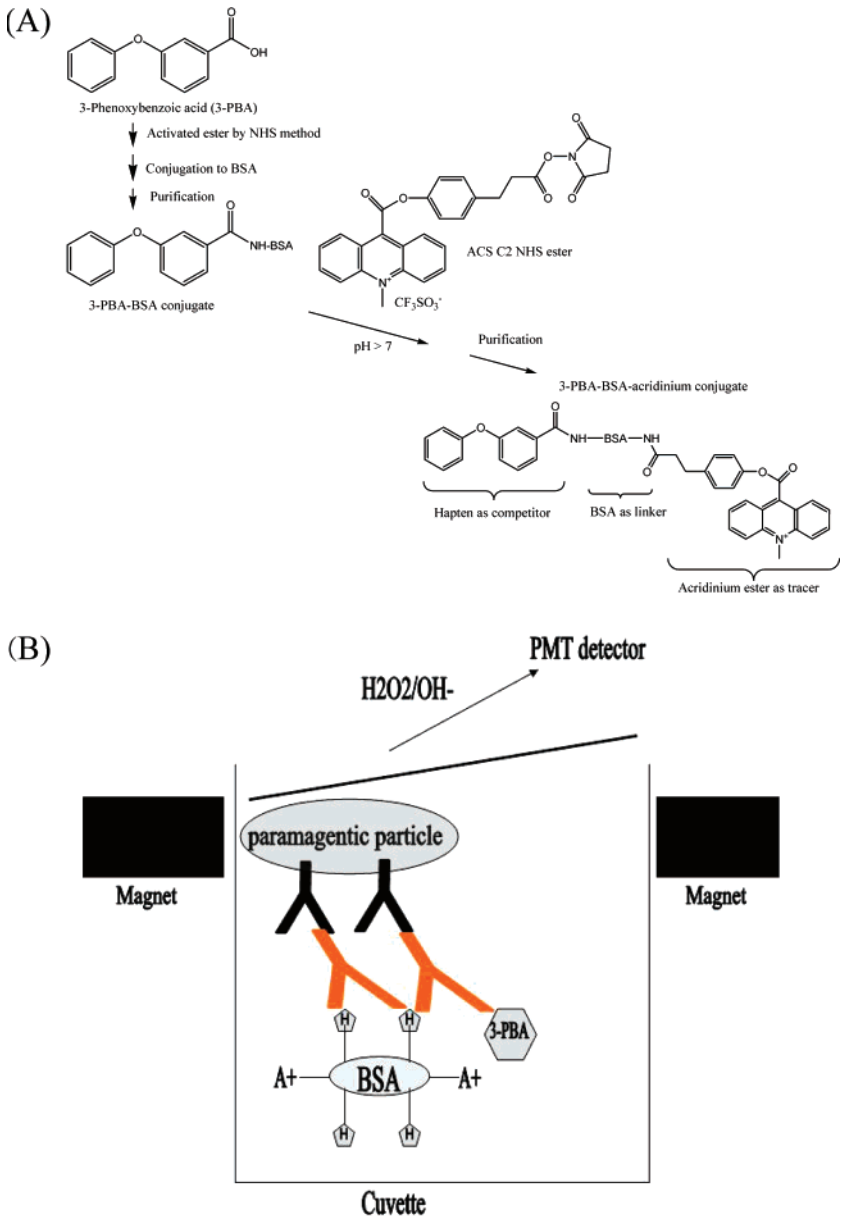


Figure 1. Scheme of the preparation of 3-PBA−BSA−A^+ conjugate (A) and the assay format (B). H, competing hapten; A^+, acridinium ester; Y, primary polyclonal antibody specific to 3-PBA; paramagnetic particle-Y, secondary antirabbit IgG-coated paramagnetic particle; PMT, photomultiplier tube, after magnetic separation and washing (solid line), chemical reaction generates light followed by measurement by PMT.

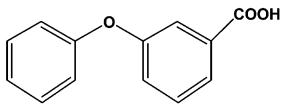
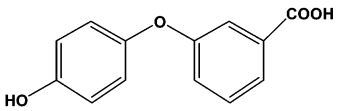
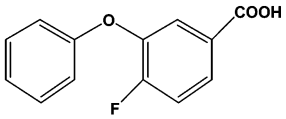
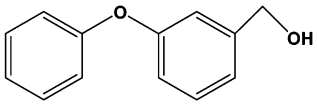
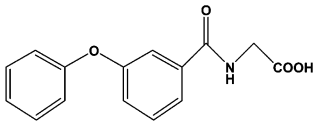
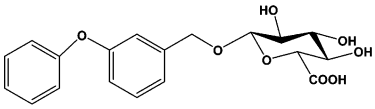
homogenized. Each C18 column was preconditioned with 3 mL of MeOH, 3 mL of 0.1% TFA in 1:1 MeOH/deionized water, and 3 mL of 0.1% TFA in deionized water. Each urine sample (0.5 mL) was then loaded onto the column and eluted with 2−4 mL/min flow rates. Columns were subsequently washed with 10 mL of 0.1% TFA in water and 3 mL of 60:40 MeOH/water with 0.1% TFA. After drying under high vacuum for 15 min, each column was finally eluted with 3 mL of MeOH or ACN (for further cleanup with liquid−liquid extraction). The eluate was evaporated to dryness under a stream of nitrogen gas, dissolved in 0.5 mL of 10% MeOH in PBS, and diluted up to 50 times with PBST prior to assay.

Further Cleanup with Liquid−Liquid Extraction (LLE) of the Acetonitrile Eluate. For further cleanup, the ACN eluate was washed twice with 2 mL of hexane to remove possible lipid coextractants. The ACN layer was then evaporated to dryness under a gentle stream of nitrogen gas, dissolved in 0.5 mL of 10% MeOH in PBS, and then diluted up to 20 times prior to assay.

Cleanup with Mixed-Mode SPE (C8 + Strong Anion Exchange, 200 mg Screen-A Tube). Each mixed-mode column was preconditioned with 1 mL each of MeOH, water, and sodium acetate buffer (pH 4). Urine sample (0.5 mL) was loaded onto the column, and an equal volume of sodium acetate buffer was added to it. Each column was subsequently washed with 2 mL of deionized water and 2 mL of MeOH. After drying under high vacuum (10 inches Hg) for 10 min, each column was finally eluted with 3 mL of 1% acetic acid in a 70:30 hexane/ethyl acetate mixture. The eluate was evaporated to dryness as described above, dissolved in 0.5 mL of 10% MeOH in PBS, and then diluted up to 5 times with PBST prior to assay.

Data Analysis. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which

Table 1. CRs (%) of Possible Metabolites with a Phenoxybenzyl Moiety in the 3-PBA Immunoassay

Compound	Chemical structure	CR (%)
3-Phenoxybenzoic acid (3-PBA)		100
4′-Hydroxy-3-phenoxybenzoic acid (4′-OH-3-PBA)		126
4-Fluoro-3-phenoxybenzoic acid (F-3-PBA)		72
3-Phenoxybenzyl alcohol (3-PBAlcohol)		0.8
Glycine conjugate of 3-PBA (3-PBA-Glycine)		2.4
Glucuronide conjugate of 3-PBAlcohol (3-PBAlc-glucuronic acid)		0.2

were fitted to a four-parameter logistic equation, $y = (A - D)/[1 + (x/C)^B] + D$, where A is the maximum signal at no analyte, B is the curve slope at the inflection point, C is the IC_{50}, and D is the minimum signal at infinite concentration. The IC_{50} value was expressed as the sensitivity of the immunoassay. Signal-to-noise (S/N) ratio was calculated from maximum signal (A)/minimum signal (D) from the above equation.

RESULTS AND DISCUSSION

Preparation of Acridinium Ester-Labeled Hapten−Protein (3-PBA−BSA−A^+) Conjugates and Their Comparison for the Immunoassay. Three proteins, BSA, HRP, and OVA, were evaluated as protein linkers between 3-PBA and the chemiluminescent acridinium ester. Neither 3-PBA−HRP−A^+ nor 3-PBA−OVA−A^+ conjugates produced standard curves with a low nonspecific binding. In addition, neither of these conjugates resulted in an assay more sensitive than the previously reported ELISA (data not shown). However, the 3-PBA−BSA−A^+ conjugate functioned well and its use generated a calibration curve with a S/N for required sensitivity and an optimal sigmoidal curve shape for the required dynamic assay range (Figure 2). The MALDI-TOF-MS results showed the successful conjugation of 3-PBA to BSA and acridinium ester tracer to the 3-PBA−BSA conjugate with a molar ratio of 16:1:5 3-PBA/BSA/A^+. BSA provides a convenient conjugation linker with multiple primary amines that can be used as conjugation points for NHS-esters of acridinium and 3-PBA and facilitates the easy separation of conjugate from unreacted chemiluminescent label/3-PBA with size exclusion methods. HRP and OVA may also be suitable linkers, but further studies would be needed to determine the cause of the high background.

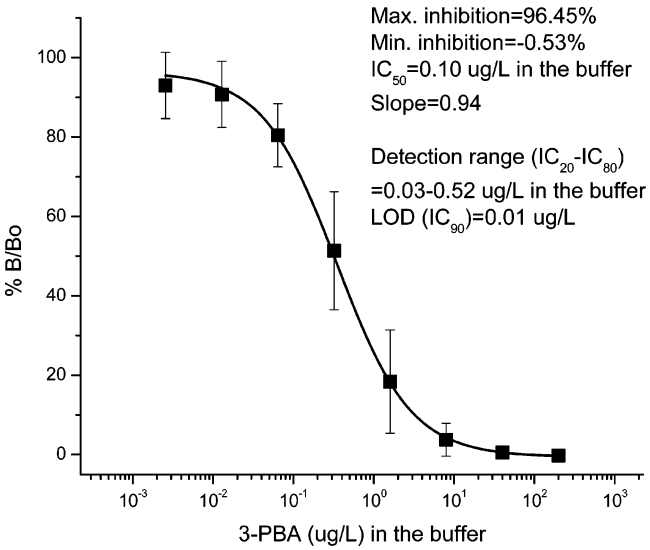


Figure 2. Chemiluminescent immunoassay (CL) inhibition curves for 3-PBA. Immunoreagent concentrations: antiserum no. 294, 1/48 000 (final dilution in cuvette); 3-PBA−BSA−A^+ conjugate, 1/72 000 (final dilution in cuvette). Calibration standards were prepared in PBST. The standard curve represents the average of 10 curves (±standard deviation) plotted from data obtained for 3 months. LOD, limit of detection.

Assay Optimization for the Immunoassay. *Optimal Concentration of Immunoreagent.* In order to determine the optimum concentrations of the 3-PBA−BSA−A^+ conjugate and Ab 294 for the assay, the effects of different concentrations of the immunoreagents on the assay were evaluated. Although 3-PBA−BSA−A^+ conjugate (5 mg/mL) showed the best sensitivity at a maximum 1 152 000-fold dilution under the conditions of certain concentrations of antibody, the dilution of 1:72 000 provided the highest S/N ratio of 8 and so was selected for further study (Supporting Information Figure 1A). As a maximum concentration, the 48 000-fold diluted antibody provided a S/N ratio of 16 as well as good sensitivity and was selected for use in the assay (Supporting Information Figure 1B).

Assay Buffer and Incubation Conditions. The buffer conditions were originally optimized for a 3-PBA ELISA format based on a hapten-coated microplate, so it was necessary to determine the optimal assay buffer conditions specifically suited for the CL format described in this report. Increasing the amounts of solvents (MeOH and DMSO) in the PBST decreased assay sensitivity and suppressed assay signal (Supporting Information Figure 1, parts C and D). However, acceptable assay sensitivity was observed when the proportion of added solvents was less than 5% of the total volume.

Buffers containing different protein "blockers" such as casein and BSA or a surfactant (Tween 20) were evaluated for their abilities to minimize the nonspecific binding. The standard buffer used for automated CL by our group, potassium phosphate with 0.5% casein, was again demonstrated to produce calibration curves with a S/N ratio of 9 (Supporting Information Figure 1E). On the other hand, a hook effect was often observed with this assay buffer resulting in a markedly reduced signal level when the 3-PBA concentration was zero (Supporting Information Figure 2). This hook effect could be caused by decreased saturation of the antibody binding sites with competing 3-PBA−BSA conjugate, thus inhibiting the formation of the immunocomplex and making this buffer a poor selection for the assay described here. After continued evaluation, PBS added with Tween 20 was observed to eliminate the "hook effect" and provided a S/N of 160. The optimum assay buffer was determined to be 0.15 M PBS, pH 7.5 plus 0.05% Tween 20.

Assay sensitivity was further enhanced by preincubating the antibody and 3-PBA−BSA−A^+ prior to adding the 3-PBA returning the IC_{50} value to that observed when PBS containing casein was used without the "hook effect" (Supporting Information Figure 3). Optimum incubation conditions were observed to be as follows: 30 min preincubation of 3-PBA−BSA−A^+ and Ab 294 followed by a 30 min incubation with 3-PBA in sample or calibrator. Although assay IC_{50} values were not affected by higher ionic strength (Supporting Information Figure 1F), an absorbance reduction was observed for 2× PBS (0.3 M) that was likely the result of suppressed antibody−antigen binding interaction. This result suggests that the assay may be sensitive to the ionic strength changes in urine samples; therefore, a dilution or analyte purification/enrichment method (SPE, LLE, etc.) is recommended, the latter being preferable to minimize the dilution of analyte. Finally, the effect of varying the time for the competition incubation on assay sensitivity was evaluated with 0.5, 1, and 3 h and overnight time points. While assay sensitivity was not significantly different for the competition times tested, the maximum luminescent signal increased with increasing incubation times (Supporting Information Figure 1G). The optimized competition incubation time was observed to be between 0.5 and 1 h for the assay described here. The optimized competitive immunoassay had a linear range (IC_{20-80}) of 0.03−0.52 μg/L 3-PBA and an IC_{50} value of 0.1 μg/L 3-PBA. The LOD in buffer was calculated as 0.01 μg/L, the IC_{10} value (Figure 2).

The optimized chemiluminescent paramagnetic particle-based immunoassay used 3-PBA−BSA−A^+ conjugate at a dilution of 1/72 000, Ab 294 at a dilution of 1/48 000, and antirabbit IgG paramagnetic particles at a dilution of 1/4.

Cross-Reactivity (CR). All reported possible metabolites derived from the phenoxybenzyl moiety of pyrethroids were evaluated for CR in this report (Table 1). Since the antibody was generated against a hapten, 3-(3-(2-oxoethoxy)phenoxy)benzoic acid that was synthesized to preserve the carboxylic acid (−COOH) group of 3-PBA, this immunoassay showed broad specificity for 3-PBA (100%), 4′-OH-3-PBA (121%; which is further metabolized from 3-PBA), and F-3-PBA (72%) which is derived from cyfluthrin. As expected, little CR was observed for 3-PBA-glycine, 3-phenoxybenzyl alcohol, and 3-phenoxybenzyl alcohol−glucuronide conjugate where the −COOH group is no longer exposed. Unlike chromatographic separation and detection methods that can provide nonspecific detection of the separated analyte, this immunoassay can be used to monitor 4-OH-3-PBA and F-3-PBA as well as 3-PBA. As expected, since there is no −COOH group exposed in the parent compounds, the recognition of the antibody for the native pyrethroid insecticides is negligible.[29] The IC_{50}s of the parent pyrethroid insecticides, esfenvalerate, cypermethrin, permethrin, deltamethrin, or cyfluthrin were over 10 000 μg/L, and the corresponding CRs are close to 0%.

Sample Cleanup of Urine. Because simple 50-fold dilution of the urine sample showed various signal differences at low concentration from individual urine samples (Supporting Information Figure 4), it was concluded that a sample cleanup method that reduced problems associated with urine matrix prior to immunoassay was needed. Although the automated CL immunoassay results coupled to C18 SPE sample cleanup facilitated acceptable recoveries with a 1:50 dilution of eluate (calculated on the basis of the original urine volume loaded onto SPE) (Table 2), the assay was limited to quantify those urine samples with concentrations >5 μg/L. The additional LLE step with hexane to remove possible lipid coextractants reduced the matrix-associated signal at a 1:20 dilution to approximately 1.5 μg/L. Sample preparation with mixed-mode SPE strong cation exchange gave excellent sensitivity and good average recovery of 91.7% for all tested concentrations with a 1:5 dilution prior to immunoassay (Table 2), and mixed-mode (C8 + strong anion exchange) solid-phase extraction of samples facilitated measurements of 3-PBA levels as low as 1 μg/L in urine with the immunoassay described here.

Assay Comparisons. With the spiked samples prepared by the mixed-mode SPE, the automatic luminescent paramagnetic particle-based immunoassay showed a tendency for underprediction of 3-PBA (Figure 3A, slope = 0.73) compared to the ELISA (Figure 3A, slope = 1.05). This might result from unresolved matrix effects or an unidentified systematic error. The assay

Table 2. Recovery of 3-PBA from Spiked Urine Samples, Prepared by C18 SPE and Mixed-Mode SPE (C8 + Strong Anion Exchange)

	C18 SPE			mixed-mode SPE
3-PBA spiked (μg/L)	urine no. 1 SPE/50-fold diln.	urine no. 2 SPE/50-fold diln.	urine no. 2 SPE/LLE with ACN and hexane/ 20-fold diln.	urine no. 2 SPE/5-fold diln.
0	3.17 ± 0.26	3.87 ± 0.03	1.39 ± 0.18	0.65 ± 0.10
1	*a*	*a*	*a*	1.21 ± 0.18 (121% ± 17.43%)
3	*a*	*a*	*a*	2.71 ± 0.24 (90% ± 7.99%)
5	4.55 ± 0.38 (91% ± 7.61%)	5.75 ± 0.93 (115% ± 18.73%)	5.25 ± 0.39 (105% ± 7.76%)	3.85 ± 0.18 (77% ± 3.62%)
10	*a*	*a*	*a*	7.85 ± 0.23 (79% ± 2.12%)
15	14.39 ± 1.29 (96% ± 8.62%)	13.81 ± 1.97 (92% ± 13.15%)	11.08 ± 0.93 (74% ± 6.25%)	*a*

[a] Not tested.

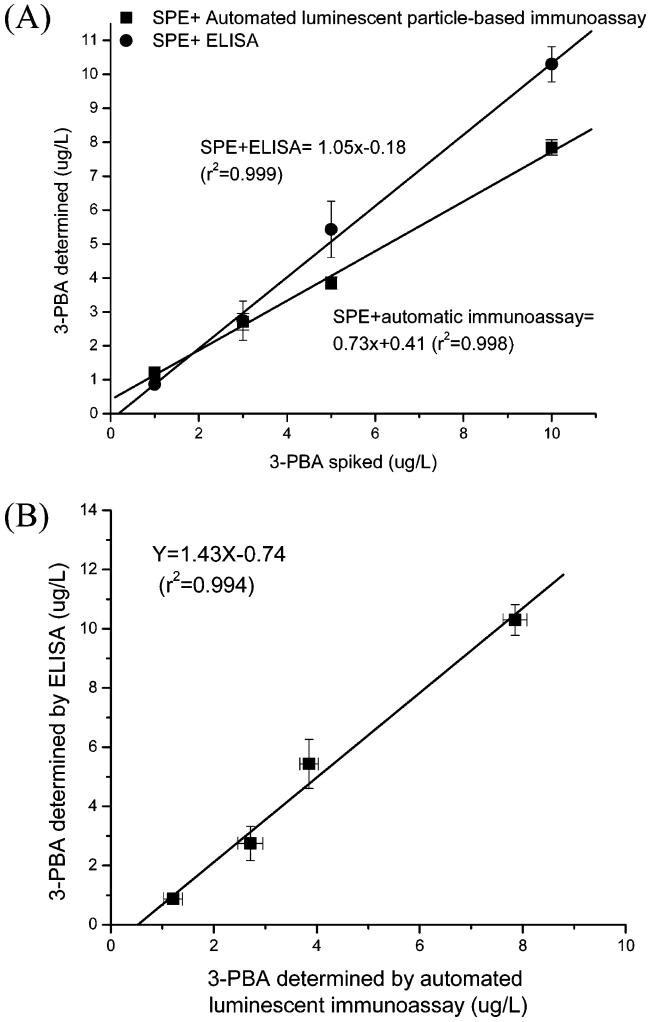


Figure 3. Correlations between the spiked and measured concentrations of 3-PBA for ELISA and automated immunoassay coupled with SPE (A) and between the concentrations measured by the automated immunoassay and ELISA (B). The ELISA was performed according to the method reported previously (ref 29) after the mixed-mode SPE for sample preparation.

comparison between the automated immunoassay and ELISA was achieved using the mixed-mode SPE method to minimize the matrix effect of urine samples which were spiked with 0−10 μg/L 3-PBA. Linear regression results showed a good correlation (r^2 = 0.994) with a slope of 1.43 (Figure 3B). These results demonstrate that the assay is appropriate for the detection of 3-PBA at low levels commonly found in urine samples.

CONCLUSION

The present report describes the successful development of a sensitive, automated CL immunoassay for use as a rapid high-throughput tool for the detection of 3-PBA in the urine of exposed human populations. This assay featured improved sensitivity with a low IC_{50} value of 0.1 μg/L for 3-PBA in buffer and an improved dynamic range of 0.03−0.52 μg/L, when compared to the previously reported microplate-based ELISA. In this case, the improved sensitivity likely results from the high affinity of antibody paired with a sensitive chemiluminescent probe and a reliable automated assay platform for liquid handling, magnetic separation, and data collection. Preincubation of the CL probe with antibody and the fact that binding events are occurring in solution rather than at a surface also likely contributed to the sensitivity of the assay. On the basis of the observed cross-reactivities, the assay can broadly monitor F-PBA and 4-OH-3-PBA as well as 3-PBA for studies monitoring human exposure to pyrethroid insecticides. The simple coupling method to prepare the conjugate containing both a competitive hapten and a signal reporter and the assay format using the automatic analyzer can be applied to other urinary biomarkers such as DCCA, the specific metabolite of some pyrethroids such as cypermethrin, permethrin, and cyfluthrin, to provide more information on human exposure to pesticides of interest.[30] This collection of urinary biomarkers together with appropriate meta data can provide information to further identity whether original exposures were to parent compounds or breakdown products themselves.

For optimum immunoassay performance, a sample preparation method was needed to reduce the urinary matrix effects. A mixed-mode (both C8 and strong anion exchange) SPE was observed to facilitate measurements as low as 1 μg/L of 3-PBA in urine. In comparison with conventional coating antigen-immobilized

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Table 3. Analytical Comparison between the Conventional ELISA and Automated Chemiluminescent Magnetic Particle-Based Immunoassay for 3-PBA

	indirect coating antigen-immobilized ELISA	automated immunoassay
immunoreagents:		
polyclonal antibody (dilution)	1:10000 (in the well)	1:48000 (in the cuvette)
competitive hapten−BSA conjugate (dilution)	1:5600 (from 2.8 mg/mL of 3-PBA−BSA conjugate)	1:72000 (from 5 mg/mL of 3-PBA−BSA−A^+ conjugate, in the cuvette)
secondary antibody (dilution)	1:3000 (from 1.1 mg/mL of IgG−HRP conjugate)	1:4 (0.1 mg IgG/mg magnetic particles/mL of antibody-coated paramagnetic particles)
total volume (μL) of the mixture of immunoreagents	100 μL in the well (sample and antibody)	450 μL in the cuvette (competitive hapten, sample, and antibody)
analytical sensitivity:		
IC_{50} (μg/L)	1.65	0.10
IC_{20-80} (μg/L)	0.20−5.0	0.03−0.52
LOD (μg/mL)	0.1	0.01
competition time between hapten and analyte to bind to antibody	1 h (the mixture of Ab and sample in 3-PBA−BSA coated well)	1 h (30 min for premixing Ab and sample, follow by 30 min of incubation after addition of 3-PBA−BSA−A^+)
analytical time after a competition step	96 results in a 96-well microplate in approximately 2 h with incubation times for the secondary antibody and color development	130 results in 1 h with the first result available in 15 min and subsequent results every 20 s
analytical procedure	hand-operated	automatic

microplate-based ELISA developed for 3-PBA (Table 3), the automated chemiluminescent 3-PBA immunoassay confers the following advantages: the sensitive chemiluminescent probe and specific antibody allow for sufficient reagent dilution to allow for assays approximately 20-fold more sensitive than the previous 3-PBA ELISA, thus allowing for the great economy of scale necessary for large studies; the enhanced sensitivity is advantageous as samples may be diluted more to decrease matrix interferences, while still maintaining the desired limit of quantitation; the ACS:180 provides the first result within 15 min of run time and produces up to 130 results per hour. As such, employing a fully automated analyzer after a competitive incubation step supports a high-throughput assay for population-based human studies which require the analyses of many samples by broadly applicable methods such as that described in this report. In addition, this classical platform is used with other markers such as hormones indicative of reproductive health.[31] With the ability to measure pesticide metabolites, a single platform can be used to measure both exposure and effect.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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