Biological monitoring of 3-phenoxybenzoic acid in urine by an enzyme-linked immunosorbent assay

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Abstract

An enzyme-linked immunosorbent assay (ELISA) method was employed for determination of the pyrethroid biomarker, 3-phenoxybenzoic acid (3-PBA) in human urine samples. The optimized coating antigen concentration was 0.5 ng/mL with a dilution of 1:4000 for the 3-PBA antibody and 1:6000 for the enzyme conjugate. Urine samples were hydrolyzed with concentrated hydrochloric acid; extracted with dichloromethane and solvent-exchanged into a methanol/buffer solution, prior to analysis in a 96-microwell plate immunoassay. Quantitative recoveries of 3-PBA were obtained for fortified urine samples by ELISA (92±18%) as well as by gas chromatography/mass spectrometry (GC/MS) (90±13%). The overall method precision of these samples was within ±20% for both the ELISA and GC/MS methods. Analytical results from over one hundred urine sample showed that the ELISA and GC/MS data were highly correlated, with a correlation coefficient of 0.95. At the 10 ng/mL comparative concentration level, the false positive rate was 0% and the false negative rate was 0.8 % for ELISA when using GC/MS as the reference method. The ELISA method has a suitable low detection limit for 3-PBA to assess pyrethroid exposures in non-occupational settings.

Key Words: 3-phenoxybenzoic acid; biomonitoring; urinary biomarker; enzyme-linked immunosorbent assay.

1. Introduction

Natural pyrethrins and their synthetic analogues, pyrethroid pesticides, are neutrotoxins, used to control insect infestations in various commercial applications and residential settings. Natural pyrethrins, isolated from chrysanthemum flowers, have high insecticidal activity but are unstable in sunlight [1]. Pyrethroids are based on pyrethrin structures that have been modified to retain insecticidal activity and increase stability toward sunlight for longer residence times in the environments. There is an increasing trend of indoor pyrethroid usage since the federally mandated phase-outs of most residential uses of the organophosphate (OP) pesticides chlorpyrifos and diazinon [2, 3]. Pyrethroids are usually less volatile than OP pesticides and tend to adsorb highly onto materials such as carpets, fabrics, and dust [4]. They have been identified in multiple environmental media (air, dust, and soil) collected from non-occupational, residential settings [4]. Human exposures to pyrethroids have been reported in the literature, including exposure to infants and other vulnerable population segments [5-7]. Routes of exposure include inhalation, dermal absorption, and direct or indirect ingestion.

Pyrethroids contain the common chemical structural features of an acid moiety, a central ester bond, and an alcohol moiety (Figure 1). The compounds are metabolized by hydrolytic cleavage of the ester bond. A number of pyrethroids such as permethrin, cypermethrin, deltamethrin, fenvalerate, and sumithrin are metabolized to 3-phenoxybenzoic acid (3-PBA). This urinary metabolite is a useful generic biomarker for assessing human exposures to pyrethroids [4, 8, 9]. Instrumental analytical methods have been developed for determining 3-PBA in urine at low or sub ppb levels [9-11]. However, hydrolysis, extraction, derivatization,

and clean up procedures are necessary for gas chromatography/mass spectrometry (GC/MS) detection. Alternative lower-cost bioanalytical approaches using enzyme-linked immunosorbent assays (ELISAs) have been reported for several pesticides including pyrethroids and their metabolites [12-18]. An ELISA for determining 3-PBA in human urine samples has been reported; however, only two urine samples from exposed workers were analyzed [13].

Reported here is the evaluation of this 3-PBA ELISA for application to exposure monitoring. Assay conditions were optimized to detect low level samples as would be encountered in a nonoccupational exposure study. The final assay was compared to GC/MS for urine samples in several key areas: accuracy, precision, sample throughput, detection limits, as well as false negative and false positive rates. The 3-PBA ELISA method employed a polyclonal antibody and a coating antigen in a 96-microwell format as previously reported [13]. Sample preparations for the ELISA method consisted of hydrolyzing/extracting the urine sample in acidic dichloromethane (DCM), exchanging the DCM extract into methanol, and diluting the methanol extract for ELISA. The ELISA method was applied to more than 100 human urine samples collected in residential settings and compared with GC/MS results for the same sample set.

2. Experimental

2.1 Urine sample collection and preparation

Urine samples collected from two observational measurement field studies [Pesticide Exposure of Preschool Children Over Time (PEPCOT) and Children's Total Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP)] were obtained [19, 20]. The first morning void urine samples were collected from each child during a 24-hr sampling period from the PEPCOT study. Up to six spot urine samples (i.e., the first morning void, after lunch, and before bedtime) were collected from each child over the 48-hr sampling period. Urine samples were kept refrigerated after each collection and at the end of the 24-h or 48-h collection periods were packed in dry ice and shipped to Battelle's Columbus Laboratories for analysis. Upon receipt of samples, the samples were frozen and stored at < -70° C till analysis.

An aliquot (10-14 mL) of each urine sample was mixed with 0.5 mL of concentrated HCl and 1 mL of DCM. The sample vial was sealed with a Teflon-lined cap and heated in an oven at 80±5°C for one hour. After heating, the sample was cooled to room temperature and an aliquot (10 mL) of 20% sodium chloride solution was added to the mixture. The resulting solution was extracted with DCM (2 x 15 mL) in a separatory funnel and dried over anhydrous sodium sulfate. The DCM extract was solvent-exchanged into methanol for subsequent ELISA analysis.

A separate aliquot of each urine sample was prepared for the GC/MS analysis. The same procedures described above were used with the exception of the DCM/methanol solvent exchange step. A known amount of the internal standard (IS), dicamba-d_3 was added to each concentrated DCM extract. The DCM extract was methylated with diazomethane in ethyl ether generated *in situ* from Diazald, carbitol, and 37% aqueous potassium hydroxide. The methylated

sample extract was solvent exchanged into hexane and processed through a pre-packed 1g, Florisil solid-phase extraction (SPE) column (Bakerbond) that was conditioned with 50% ethyl ether in hexane (6 mL), followed by 100% hexane (6 mL). The SPE column was eluted with 18 mL of 50% ethyl ether in hexane, and the collected fraction was concentrated to 1 mL for subsequent GC/MS analysis.

2.2 ELISA procedures

The ELISA urine sample analyses were performed using a 96-microwell format. All standards and control solutions were prepared in a methanol extract containing 10% hydrolyzed drug free urine (hDFU) and stored at -20±5°C for the duration of the study. The hDFU methanol extract was prepared by hydrolyzing 90 mL of DFU (American Biological Technologies Inc., Sequin, TX) using the procedures described in Section 2.1. The resulting methanol extract (10 mL), designated as hDFU, contained a 9-fold concentrated urine matrix (90 mL DFU/10 mL methanol). A 10 to 1 dilution was performed on the hDFU methanol extract and designated as a 10% hDFU (representing 0.9 fold urine concentrate methanol extract). This 10% hDFU methanol extract was used for the preparation of standards, and control solutions for the ELISA. An additional 1:5 dilution with phosphate buffered saline (PBS) was performed during the assay as described below.

The anti-rabbit 3-PBA antibody (Ab294) and the coating antigen were provided by Dr. Bruce D. Hammock of the University of California at Davis [13]. The coating antigen and the commercial enzyme conjugate secondary antibody (goat anti-rabbit IgG peroxidase conjugate,

Sigma Chemical Company, St. Louis, MO) along with the anti-PBA antibody were titrated at various concentrations to achieve optimal assay conditions.

A 96-well Nunc Maxisorb plate (Nalge Nunc, Milwaukee, WI) was coated with (100 µL/well) coating antigen at 0.5 ng/mL in 0.05M carbonate bicarbonate buffer, pH 9.6 (Sigma, St. Louis, MO) and incubated overnight at 5±3°C. Each plate was washed six times with (300 µL/well) 10 mM phosphate buffered saline, pH 7.4, 0.05% Tween 20 (PBST, Sigma, St. Louis, MO). The plates were rotated 180^{o} between wash cycles to minimize well-to-well variability. The plates were further dried by tapping on absorbent paper. An aliquot (200 µL/well) of a blocking buffer solution, 0.5% bovine serum albumin (BSA) in PBS (Sigma, St. Louis, MO) was added to each plate and incubated for 30 minutes at room temperature. The plates were then washed six times with PBST to remove excess blocking agent.

The 3-PBA calibration standard solutions, quality control (QC) spiked urine and study urine samples were analyzed in triplicate for each assay. Each standard and sample was in methanol containing the same concentration of urine to normalize any matrix effect. A 1:5 dilution with PBS for all standards, QC samples, and urine samples was performed in the microwells during the assay procedure. A 10-point (0.005-100 ng/mL) calibration curve was generated for each assay plate. The highest standard solution was prepared at 100 ng/mL and the remainder of the standard solutions was prepared by a serial 1:3 dilution from the highest standard solution to concentrations of 33.3, 11.1, 3.70, 1.23, 0.411, 0.137, 0.046, 0.015, and 0.005 ng/mL. A 0 ng/mL standard in 80% PBS/20% methanol with 10% hDFU was also prepared for each assay to confirm that the use of hDFU does not contribute to 3-PBA response

in the standard curve. Individual aliquots (50 µL/well) of the standard solutions, urine samples, and QC samples prepared in 80%PBS/20% methanol were placed in triplicate microwells. An aliquot (50 µL) of the anti-PBA antibody (1:4000) in PBS with 0.2% BSA was added to each microwell except those used as instrument blanks which received 50 µL of PBS with 0.2% BSA . Plates were incubated for 1 hour at room temperature on an orbital shaker at 140 rpm. Excess reagent not bound to the plate was removed by washing six times with PBST as described above. An aliquot (100 µL) of goat anti-rabbit IgG peroxidase conjugate (Sigma, St. Louis, PA) at a 1:6000 dilution in PBST was added to each microwell. Plates were again incubated for 1 hour at room temperature on the orbital shaker (140 rpm). Excess conjugate was removed by washing with PBST. A 100 µL aliquot of 3,3',5,5'- tetramethylbenzidine (TMB), peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) was added to each microwell. Following a15 minute room-temperature incubation on the orbital shaker, color development was stopped with the addition of 50 µL of TMB stopping solution (Kirkgaard & Perry, Gaithersburg, MD) to each microwell. Absorbance was measured at 450 nm on a SpectroMax microplate reader (Sunnyvale, CA). Data processing was performed with SoftMax Pro7 software version 4.6 interfaced to a personal computer using a 4-parameter curve fit.

2.3 GC/MS procedures

The sample extracts and standard solutions were analyzed by 70 eV electron impact (EI) GC/MS. A Hewlett-Packard GC/MS was operated in the selected ion monitoring (SIM) mode. Data acquisition and processing were performed with a ChemStation data system. The GC column was a DB-5 fused silica capillary (60m x 0.32 mm, 0.25 µm film thickness). Helium

was used as the GC carrier gas. Following injection, the GC column was set at 90°C for 2 min, temperature programmed to 290°C at 8°C/min., and held at 290°C for 5 min. Peaks monitored were the molecular ion peaks and their associated characteristic fragment ion peaks (197, 198, 228, 229 for 3-PBA, 215, 216, 246, 247 for FPBA, and 237 and 239 for the IS, dicamba-d_3). Identification of the target analytes was based on their GC retention times relative to the IS and the relative abundances of the monitored ions. Quantification was performed by comparing the integrated ion current response of the target ions to those of the IS. The average response factors of the target ions were generated from the standard calibrations [4]. In brief, the Rf value was obtained using $Rf = (A_S/A_{IS}) * (C_{IS}/C_S)$ from the analyses of standard solutions used for generating the calibration curve. Note that A_S and A_{IS} refer to the area counts of the quantification ions of target analytes and the IS, respectively, and C_S and C_{IS} are the concentration values of target analytes and the IS, respectively. The concentration of target analytes (C_S) in the samples was obtained from $(A_S/A_{IS}) * (C_{IS}/Rf_{avg})$, where the Rf_{avg} value was the average Rf value generated from the analyses of standard solutions.

3. Results and discussion

3.1 Method evaluation for 3-PBA ELISA

The anti-PBA antibody exhibited no cross reactivity toward the parent pyrethroids (cypermethrin, cyfluthrin, deltamethrin, esfenvalerate, permethrin). However, there was high cross-reactivity (72%) for 4-fluoro-3-phenoxybenzoic acid (FPBA), a metabolite of cyfluthrin, a common pyrethroid with fluorine substituted to the alcohol moiety (Figure 1). The high-cross

reactivity to FPBA offers an advantage for using this ELISA as a potential biomonitoring tool for common urinary pyrethroid metabolites in humans. Checkerboard titration experiments were performed to determine the optimal concentrations of anti-PBA antibody, coating antigen, and the antibody-enzyme conjugate. The optimal conditions established for the 3-PBA ELISA were 0.5 ng/mL of coating antigen, a dilution of 1:4000 of anti-PBA antibody and a dilution of 1:6000 of the antibody-enzyme conjugate (goat anti-rabbit peroxidase). Initially, calibration curves for 3-PBA were generated in ten concentration levels ranging from 0.00256 to 500 ng/mL (1:5 dilution series) to establish the working concentration range for the 3-PBA ELISA. The range of the calibration curve was further defined as 0.005 to 100 ng/mL (1:3 dilution series) and used for all sample analyses in this study.

A typical standard calibration curve consisting of ten concentration levels (0.005 to 100 ng/mL) is shown in Figure 2. Day-to-day consistency was observed for the shape of the calibration curves. Only a trace amount of 3-PBA was present in the hDFU as determined by GC/MS. This amount did not contribute any significant response for the 0 ng/mL standard. The ELISA had a 50% inhibition (IC_{50}) value of approximately 1.5 ng/mL. The % relative standard deviation (%RSD) values of the triplicate analyses were less than 20% for the standard solutions. Day-to-day variation for the 1.0 ng/mL quality control (QC) standard was within 13.1% (1.2±0.16 ng/mL). The estimated assay detection limit was 0.2 ng/mL in the assay, representing 0.1 ng of 3-PBA in one mL of urine (a 10-fold concentration of hydrolyzed urine in methanol extract followed by a 5-fold dilution with PBS for ELISA).

Triplicate analyses were conducted for each urine sample aliquot by ELISA. The means of the triplicate values were used to calculate the final concentrations of the samples. The percent difference (%D) concentrations in duplicate aliquots of selective urine samples ranged from 7.6 to 13.8% with an average of 11±2.3%. Recoveries of the fortified urine samples ranged from 70 to 117% with an average of 92±18%. In summary, the overall method precision for the ELISA method was within ±20% and the overall method accuracy was greater than 70%.

3.2 3-PBA ELISA for human urine samples

Aliquots from more than one hundred urine samples from subsets of two observational field studies [19, 20] were prepared and analyzed by the 3-PBA ELISA method. Each plate contained ten calibration standards (0.005 to 100 ng/mL), three QC solutions, one instrument blank, as well as the urine samples. Triplicate analyses were conducted for all standards, QC solutions, and urine samples. The means of the triplicate values were used to calculate the final concentrations of the standards and/or samples. Data acceptance criteria for the 96-microwell plate assay were established and used as guidance for analysis of these samples. The four parameter curve-fit values of: (a) upper asymptote, (b) slope, (c) IC_{50}, and (d) lower asymptote were generated for each calibration curve. The average recovery of the QC solutions was 94.7±20.1 %. Table 1 summarizes the performance of the ELISA calibration curve during the study period. As shown in Table 1, the IC_{50} values ranged from 0.88 to 2.21 ng/mL with an average of 1.40±0.40 ng/mL. The square of the correlation coefficient (R^2) of each calibration curve ranged from 0.984 to 0.999. The % relative standard deviation (%RSD) values of the triplicate analyses were less than 20% for all standard solutions. If the %RSD value was greater

than 30%, the sample extracts were diluted and assayed. The ELISA derived 3-PBA concentrations ranged from <0.1 to 19 ng/mL. Among the urine samples analyzed, three urine samples had no detectable 3-PBA.

3.3 GC/MS results

The acceptance criteria for GC/MS analysis of 3-PBA and FPBA were: (1) <15% relative standard deviation (RSD) value of the average response factor of 3-PBA and FPBA to the internal standard, dicamba-d_3; (2) <20% difference value of the measured and expected value of the standard solution; and (3) 80 - 120% recovery of a matrix spiked sample. The GC/MS data met all of the QA requirements stated. The overall precision of the GC/MS method was within ±20%, with an accuracy greater than 80%. For GC/MS results, the measured concentrations ranged from <0.2 to 22.1 ng 3-PBA/mL of urine and <0.2 ng to 0.55 ng FPBA/mL of urine. Note that the estimated detection limit was based on the lowest level of the calibration standard solution (2 ng/mL) and the sample size of 10 mL of the urine. The 3-PBA was detected frequently (95% detectable rate) in the urine samples but FPBA was only detected in ten samples (8.4% detectable rate).

Quantitative recoveries were obtained in the six sets of fortified and unfortified urine samples and the average recoveries were 90±13% for 3-PBA and 95±13% for FPBA. The percent difference (%D) of duplicate aliquots of a subset of urine samples analyzed by GC/MS ranged from 0.6 to 26% for 3-PBA with an average of 12%. The %D value for FPBA could not be obtained because FPBA was not detected in this subset of samples.

3.4 Comparison of ELISA and GC/MS data

Since the ELISA method has high cross reactivity (72%) toward FPBA, the ELISA derived results (based on 3-PBA quantification) could be treated as the sum of FPAB and 3-PBA concentrations in a given urine sample. Both FPBA and 3-PBA were measured in the urine samples by the GC/MS method. For these urine samples, 3-PBA was detected in 95% of the samples while FPAB was detected in only 8.4% of the samples (10 out of 119 samples). The results indicate that FPBA was detected at a much lower rate than 3-PBA in human urine samples collected from non-occupational settings. A similar trend was reported in the National Report on Human Exposure to Environmental Chemicals from urine samples collected for the National Health and Nutrition Examination Survey (NHANES) [11]. For comparison with the ELISA, the GC/MS data were corrected for the ten samples that had detectable FPAB. Using the cross-reactivity value of 72%, FBPA concentration was converted to 3-PBA equivalent and added to the 3-PBA concentration for the sample. For any non-detects, one half of the detection limit was used for the statistical analysis. Creatinine levels were not measured in the urine samples from the PEPCOT study, due to the small individual sample volumes and the uncertainty of creatinine corrections for small children [21]. Thus, for the comparison of ELISA and GC/MS described below, we used the concentration data expressed as ng/mL of urine.

Summary statistics (sample size, arithmetic and geometric means, standard deviation, minimum, 25th percentile, 50th percentile, 75th percentile, 95th percentile, and maximum) for the ELISA and GC/MS results, expressed as PBA concentrations are shown in Table 2. The arithmetic mean values were 1.29 ng/mL in urine for the ELISA and 1.23 ng/mL for GC/MS. In

general, similar 3-PBA concentrations in the 25th, 50th, 75th, and 95th percentiles were observed in these urine samples for the two detection techniques. The Pearson correlation coefficient between the 3-PBA concentrations of the two methods for all the samples was r=0.952, which was statistically significant ($p<0.0001$). Thus, there is very strong statistical evidence of a general linear association present in the reported 3-PBA concentrations between the ELISA and GC/MS methods for these samples. Figure 3 displays the relationship between the data from the ELISA and the GC/MS methods. The data points (N = 119) for Figure 3 are summarized in Table 2 as well as the linear regression equation and the square of the correlation coefficient, R^2 ($R^2 = 0.9059$). The fitted equation is: ELISA = 0.873 x GC/MS + 0.2159 for all the data. The correlation was examined further for concentrations less than 2 ng/mL by GC/MS as the majority of the data (N = 105) were in this region. The fitted equation is ELISA = 0.9428 x GC/MS + 0.0974 and R^2 is 0.7456. The slopes in both cases are close to 1 suggesting that the results derived from the ELISA and GC/MS are not identical but similar and positively correlated. Similar sample preparation methods were involved in the ELISA and GC/MS. The same hydrolysis procedures were used for both methods. A solvent exchange step (from DCM to methanol) was required for ELISA while methylation and cleanup steps were employed prior to GC/MS. Thus, the difference observed between the two methods was mostly from the detection techniques. Overall, there was good correlation between direct ELISA and GC/MS methods for this set of urine samples. A non-significance outcome ($p=0.756$) was also observed from the paired t-test indicating that there was no significant differences in measurements between the two analytical methods (ELISA vs GC/MS) for a given sample. The false positive and false negative rates for the ELISA were also examined using results derived from the GC/MS as reference values. If the GC/MS procedure represents a standard method, then the false positive

and false negative rates for the ELISA were 2.5% and 4.2%, respectively, at the 1 ng/mL concentration level. At the 10 ng/mL concentration level, the false positive and false negative rates decreased to 0% and 0.8%. As these urine samples were collected from residential environments, only three of the 119 urine samples had 3-PBA concentrations greater than 10 ng/mL as detected by GC/MS.

As noted in Table 2, 3-PBA concentrations detected in the human urine samples are typically less than 1 ng/mL with 75th percentile concentrations of 1.03 ng/mL by ELISA and 1.05 ng/mL by GC/MS. The urine samples reported here are from pre-school aged children (<6 year). Similar concentrations were reported for children in the 6-11 age group from the NHANES study [11]. Significantly higher 3-PBA concentrations have been reported for pest control operators, greenhouse workers, and agricultural workers through occupational exposures [8]. These results suggest that the 3-PBA ELISA offers a quantitative measurement of the pyrethroid urinary biomarker in sub-ppb concentration levels to provide non-occupational exposure assessment data. This ELISA can also be used for monitoring higher-level occupational exposures by simply diluting the urine samples when necessary.

Table 3 presents the method precision, accuracy, detection limit, and sample throughput between the ELISA and GC/MS methods for monitoring 3-PBA in human urine samples. The overall method precision and accuracy were similar for the two methods. The estimated detection limit is slightly lower for the ELISA method. The estimated sample throughput by the ELISA method is about the same as the GC/MS method because similar sample preparation procedures were involved in both methods, however, a derivatization step was not required for ELISA. However, ELISA could be coupled to a more streamlined sample preparation such as

utilizing small sample size and a 96-well solid phase extraction plate format to increase overall sample throughput.

4.0 Conclusion

The 3-PBA ELISA method was evaluated and compared with GC/MS for measuring 3-PBA in urine. Results from ELISA and GC/MS methods were highly correlated for real-world samples. Linear and positive relationships were observed between the ELISA and GC/MS data for 119 human urine samples. There was no significant difference between the ELISA and GC/MS data for a given sample. In conclusion, the ELISA method could be used as an alternative analytical method for quantitatively measuring 3-PBA as a non-specific urinary biomarker for monitoring studies on pyrethroids in non-occupational settings.

Table 1. 3-PBA ELISA Calibration curve fit performance data

Assay Date	Plate No.	A	B	C	D	R^2
01/19/2005	1	0.913	1.313	1.62	0.180	0.992
	2	1.148	0.944	1.27	0.167	0.989
01/20/2005	1	0.961	1.132	1.45	0.182	0.997
	2	0.985	1.25	1.91	0.203	0.992
01/21/2005	1	1.207	1.175	1.62	0.237	0.991
	2	1.077	1.298	2.21	0.229	0.997
01/27/2005	1	1.076	0.991	1.45	0.181	0.997
01/31/2005	1	1.037	1.147	1.49	0.203	0.996
02/04/2005	1	0.706	1.303	2.12	0.246	0.997
02/11/2005	1	0.796	0.982	1.27	0.201	0.998
04/21/2006	1	1.520	1.218	1.27	0.209	0.989
04/25/2006	1	1.049	1.335	1.11	0.227	0.984
	2	0.880	1.083	0.89	0.158	0.997
04/26/2006	1	0.991	1.039	0.88	0.170	0.999
	2	0.997	0.967	1.18	0.165	0.986
04/27/2006	1	0.956	1.660	1.02	0.209	0.984
04/28/2006	1	0.789	1.085	1.00	0.157	0.996
	Mean	1.005	1.172	1.40	0.196	0.993
	Std. Dev.	0.185	0.181	0.40	0.028	0.005
	%CV	18.4	15.4	28.6	14.5	0.50

Calibration curve formula: $y = ((A-D)/(1 + (X/C)^B)) + D$

A = the y-value corresponding to the asymptote at low values of the X axis; B = the curve slope; C = the X-value corresponding to the y value that is at the midpoint between A and D, showing 50% inhibition (IC_{50}); D is the y-value corresponding to the asymptote at high values of the X axis, and R = correlation coefficient of the curve fit.

Table 2 Summary statistics for the 3-PBA ELISA and GC/MS data

Summary Statistics	**ELISA, ng/mL**	**GC/MS, ng/mL**
Sample Size	119	119
Arithmetic Mean	1.29	1.23
Standard Deviation	2.54	2.77
Geometric Mean	0.64	0.57
Minimum	<0.1	<0.2
25^{th} Percentile	0.29	0.24
50^{th} Percentile	0.52	0.44
75^{th} Percentile	1.03	1.05
95^{th} Percentile	5.95	4.29
Maximum	19.0	22.1

Table 3. Comparative analyses of precision, accuracy, detection limit, and sample throughput for the ELISA and GC/MS methods for 3-PBA in urine

Method Performance	**ELISA**	**GC/MS**
Precision	±20%	±20%
Accuracy	>70%	>80%
Detection Limit	0.1 ng/mL [b]	0.2 ng/mL [b]
Sample Throughput[a]	~20/3days	~20/3days

[a] The estimated sample throughput for ELISA is based on the assumption that 10-20% of the samples outside the calibration range will be further diluted and assayed. The estimated sample throughput includes sample preparation, detection, and data analysis.

[b] Based on a10 mL urine sample size.

Figure captions

Figure 1 Chemical structures of common pyrethroid pesticides.

Figure 2 ELISA calibration curve for 3-PBA.

Figure 3 ELISA and GC/MS comparative results; all data (N = 119) shown in the upper portion; data with less than 2 ng/mL by GC/MS (N = 105) shown in the lower portion.

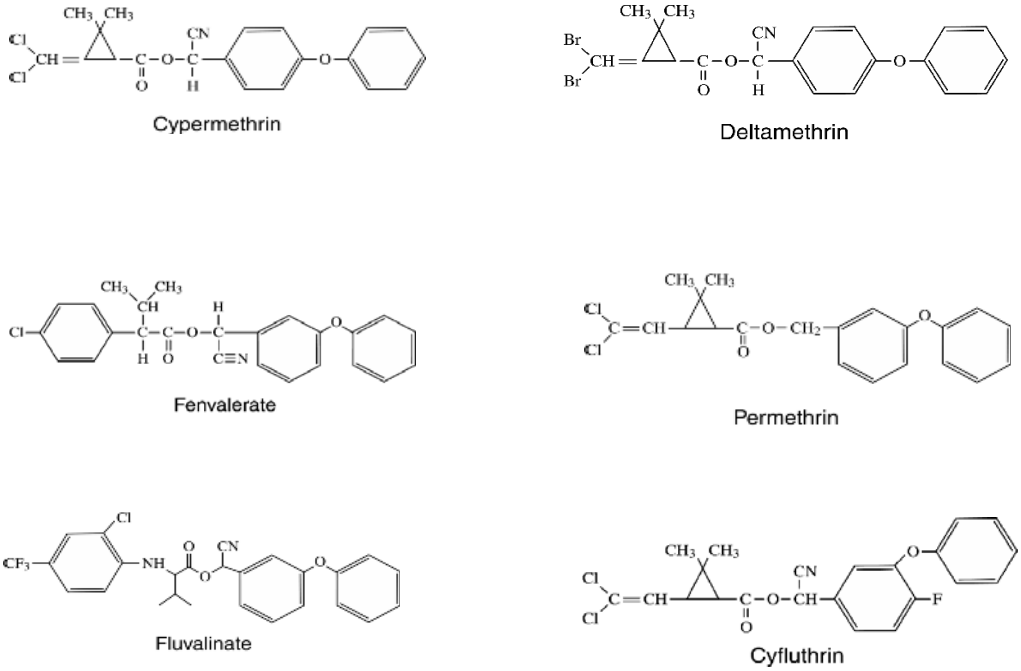

Cypermethrin
Deltamethrin
Fenvalerate
Permethrin
Fluvalinate
Cyfluthrin

Figure 1

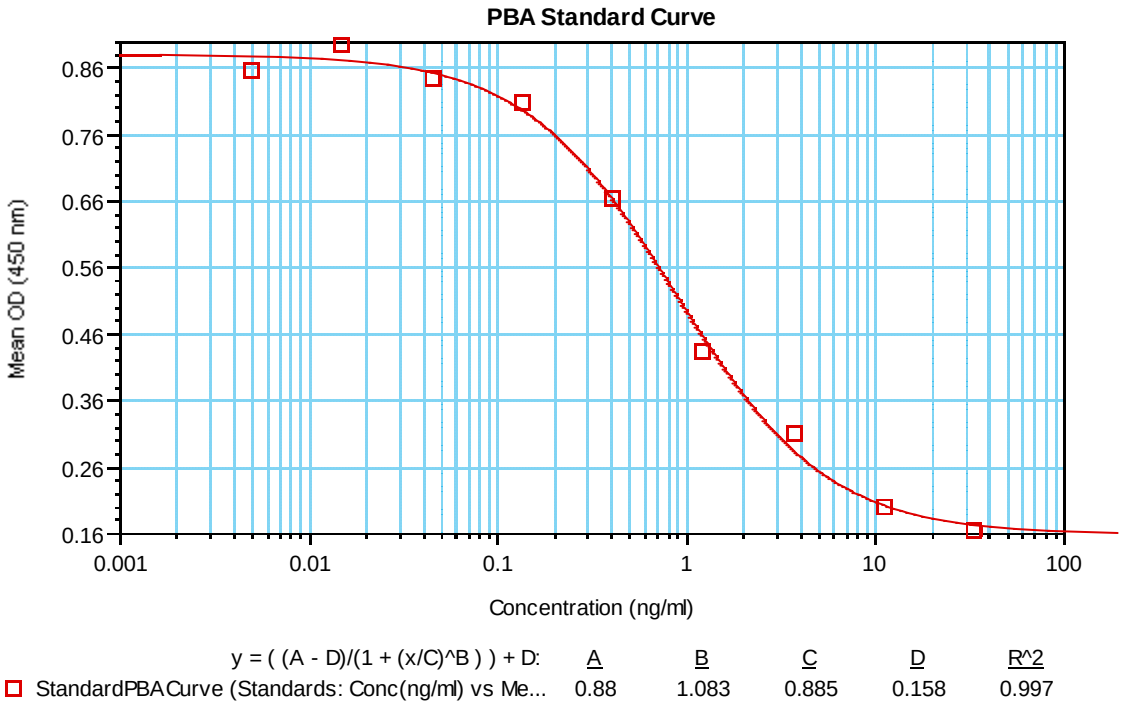
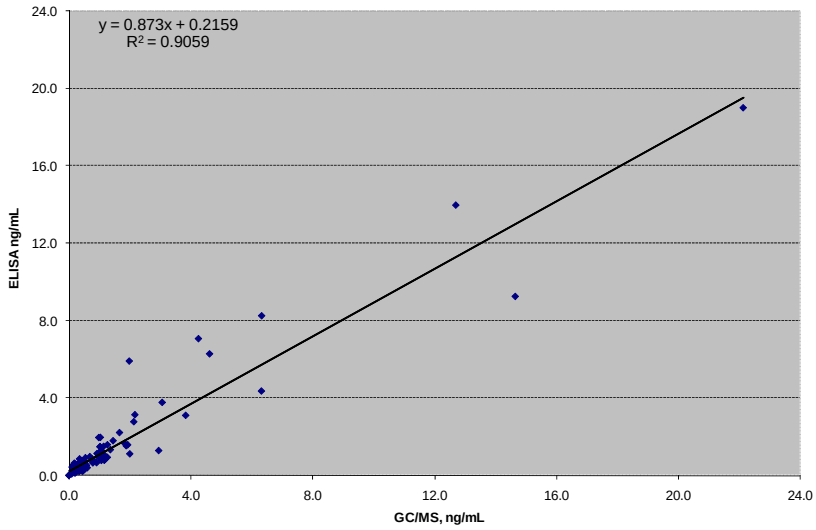

PBA Standard Curve
Mean OD (450 nm)
0.86
0.76
0.66
0.56
0.46
0.36
0.26
0.16
0.001
0.01
0.1
1
10
100
Concentration (ng/ml)
y = ((A - D)/(1 + (x/C)^B)) + D:
A
B
C
D
R^2
StandardPBACurve (Standards: Conc(ng/ml) vs Me...
0.88
1.083
0.885
0.158
0.997

Figure 2


y = 0.873x + 0.2159
R² = 0.9059
ELISA ng/mL
GC/MS, ng/mL
0.0
4.0
8.0
12.0
16.0
20.0
24.0

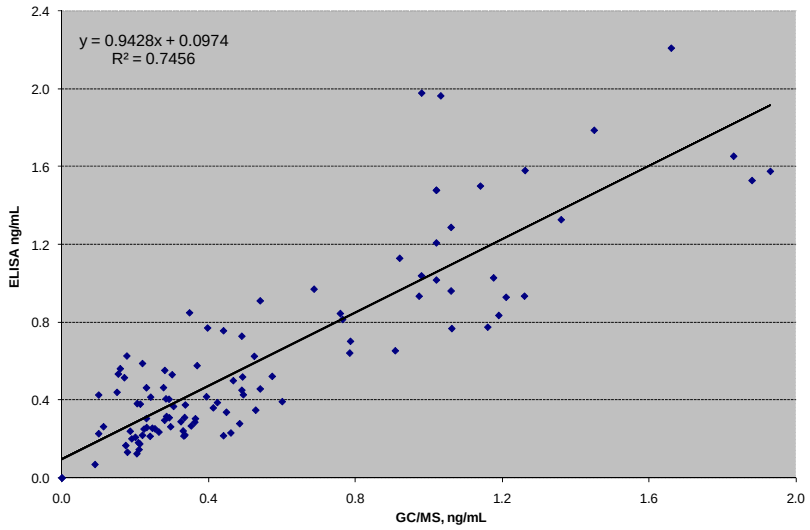

y = 0.9428x + 0.0974
R² = 0.7456
ELISA ng/mL
GC/MS, ng/mL
0.0
0.4
0.8
1.2
1.6
2.0
2.4

Figure 3

Acknowledgement

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Urine samples were obtained from two "observational research" studies as defined in 40 CFR Part 26.402. The study protocol and procedures to obtain the assent of the children and informed consent of their parents or guardians were reviewed and approved by an independent institutional review board (IRB) and complied with all applicable requirements for the Common Rule regarding additional protections for children.

References

[1] J. E. Casida. Pyrethrum flowers and pyrethroid insecticides. Environ. Health Perspect. 34:189-202, 1980.

[2] Federal Register, Volume 65, Number 235, pp 76233-76240 (December 6, 2000); C. M. Browner, Administrator, US EPA, Speech, June 8, 2000, Washington DC.

[3] Federal Register, Volume 66, Number 7, pp 1977-1981 (January 10, 2001); US EPA Headquarters Press Release, December 5, 2000.

[4] M. K. Morgan, L. S. Sheldon, C. W. Croghan, J. C. Chuang, R. A. Lordo, N. K. Wilson, C. Lyu, M. Brinkman, N. Morse, Y. L. Chou, C. Hamilton, J. K. Finegold, K. Hand, and S.M. Gordon. 2004. A pilot study of children's total exposure to persistent pesticides and other persistent organic pollutants (CTEPP). USEPA National Exposure Research Laboratory, Research Triangle Park,NC. EPA/600/R-041/193.

[5] G. S. Berkowits, J. Obel, E. Deych, R. Lapinski, J. Fodbold, Z. Liu, P. J. Landrigan, and M. S. Wolff. Exposure to indoor pesticides during pregnancy in multiethnic, urban cohort. Environ. Health Perspect. 111(1): 79-84, 2003.

[6] R W. Whyatt, D. E. Cammann, P.L. Kinney, A. Reyes, J. Ramirez, J. Dietrich, D. Diaz, D. Holmes, and F. P. Perera. Residential pesticides use during pregnancy among a cohort of urban minority women. Environ. Health Perspect. 10 (5): 507-514, 2002.

[7] U. Heudorf, and J. Angerer. Metabolites of pyrethroid insecticides in urine specimens: current exposure in an urban population in Germany. Environ. Health Perspect. 109 (3): 213-217, 2001.

[8] J. Hardt and J. Angerer. Biological monitoring of workers after the application of insecticidal pyrethroids. Int. Arch. Occup. Environ. Health 67: 492-498, 2003.

[9] T. Schettgen, H. M. Koch, H. Drexler and J. Angerer. New gas chromatographic-mass spectrometric method for the determination of urinary pyrethroid metabolites in environmental medicine. J. Chromatogr. B 778:121-130, 2002.

[10] G. Leng, K.H. Kuhn, and H. Idel. Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: application and limitations. Sci. Total Environ. 199:173-181, 1997.

[11] CDC, 2005. Fourth national report on human exposure to environmental chemicals. Center for Diseases Control and Prevention, Altanta, GA 30341. http://www.cdc.gov/exposurereport/

[12] S. K. Mak, G. Shan, H. Lee, T. Watanabe, D. W. Stoutamire, S. J. Gee, and B. D. Hammock. Development of a class selective immunoassay for the type II pyrethroid insecticides. Anal. Chim. Acta 534: 109-120, 2005.

[13] G. Shan, H. Huang, D. W. Stoutamire, S. J. Gee, G. Leng, and B. D. Hammock. A sensitive class specific immunoassay for the detection of pyrethroid metabolites in human urine. Chem. Res. Toxicol. 17:218-225, 2004.

[14] E. G. Matveeva, G. Shan, I. M. Kennedy, S. J. Gee, D. W. Stoutamire, and B. D. Hammock. Homogeneous fluoroimminoassay of a pyrethroid metabolite in urine. Anal. Chim. Acta 444: 103-117, 2001.

[15] K. C. Ahn, S. Ma, H. Tsai, S. J. Gee, and B. D. Hammock. An immunoassay for a urinary metabolite as a biomarker of human exposure to the pyrethroid insecticide permethrin. Anal. Bioanal. Chem. 384: 713-722, 2006.

[16] J. M. Van Emon. Immunochemical applications in environmental science. J. of AOAC Int. 84, 125-133, 2001.

[17] J. M. Van Emon, J. C. Chuang, R. M. Trejo, and J. Durnford. Integrating bioanalytical capability in an environmental analytical laboratory. In: Van Emon JM, ed. Immunoassay and Other Bioanalytical Techniques. New York, NY: CRC Press, Taylor and Francis, 2007; pp. 1–43.

[18] J. M. Van Emom, J. C. Chuang, K. Dill, and K. H. Xiong. Immunoassays and biosensors. In: Tadeo JL, ed. Analysis of Pesticides in Food and Environmental Samples. New York, NY: CRC Press, Taylor and Francis Group, 2008; pp. 95–123.

[19] N. K. Wilson, J. C. Chuang, K. M. Hand, M. Brinkman, and C. Lyu. Organophosphate and pyrethroid pesticides in environmental and children's personal samples collected at 50 homes of infants and toddlers. Presented in the Symposium of the International Society of Exposure Analysis, Philadelphia, PA, October 17-24, 2004.

[20] N. K. Wilson, J. C. Chuang, R. Iachan, C. Lyu, S. M. Gordon, M. K. Morgan, H. Ozkaynak, L.S. Sheldon. Design and sampling methodology for a large study of preschool children's aggregate exposures to persistent organic pollutants in their everyday environments. J. Expo. Anal. Environ. Epidemiol. 14: 260-274, 2004.

[21] O'Rourke, M. K., Lizardi, P. S., Rogan, S. P., Freeman, N. C., Aguirre, A., Saint, C. G. Pesticide exposure and creatinine variation among young children. J. Expo. Anal., Environ. Epidemiol. 10: 672-681 (2000).