Published in final edited form as:
Anal Methods. 2012 October 24; 4(11): 3772–3778. doi:10.1039/C2AY25642H.

An enzyme-linked immunosorbent assay for detecting 3-phenoxybenzoic acid in plasma and its application in farmers and consumers

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Abstract

The aim of this study was to identify a plasma biomarker of exposure to pyrethroid insecticides. A major metabolite, 3-phenoxybenzoic acid (3-PBA), can be detected in urine but urinary 3-PBA cannot be used to assess the active dose. The 3-PBA-adduct represents a much more persistent class of biomarkers than metabolites excreted into urine, having half lives up to several weeks or months. We developed an enzyme-linked immunosorbent assay (ELISA) for total 3-PBA including adduct formed after alkaline hydrolysis, liquid-liquid extraction (LLE) and solid phase extraction (SPE) of the sample. The developed ELISA had an IC_{50} value of 26.7 ng/mL. The intra- and inter-assay coefficients of variation (%CV) were lower than 5% and were within the optimum condition variance (OCV) range. The LLE cleanup technique satisfactorily eliminated the matrix effect from plasma samples before SPE and ELISA analysis yielding good recoveries (85.9–99.4%) with a limit of quantitation (LOQ, 5 ng/mL) that was 30- to 47-fold more sensitive than previous studies. Moreover, the developed method could separate more than 80% of 3-PBA from adduct form. The method was successfully applied to the detection of the target in real samples obtained from consumers (n=50) and farmers (n=50). To our knowledge, this is the first ELISA method for detecting 3-PBA in human plasma and applied to a field study.

Keywords

Enzyme-linked immunosorbent assay; Pyrethroid insecticides; 3-phenoxybenzoic acid; Plasma; Farmer; Consumer

INTRODUCTION

Synthetic pyrethroid insecticides in Thailand have been widely used in household products for controlling vectors such as flies, cockroaches, ants, and ticks. Importantly, national programs currently employ pyrethroids such as deltamethrin, cypermethrin and permethrin

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for controlling mosquito vectors of malaria and dengue diseases in endemic areas using techniques such as fogging and impregnated mosquito bed nets [1]. Moreover, pyrethroids such as cypermethrin have also been used to boost agricultural production [2].

The synthetic pyrethroid insecticides have been associated with acute toxicity (overexposure) such as headache, dizziness, nausea, irritation of the skin and nose, and paraesthesia [3, 4]. Furthermore, chronic toxicity such as lymph node and splenic damage [5], suppressive effect on the immune system [6] and hormone disruption [7] as well as carcinogenesis have been noted. After human exposure to pyrethroids, these compounds are absorbed into the serum and hydrolyzed by esterase enzymes, mainly in the liver to 3-phenoxybenzyl alcohol or 3-phenoxybenzaldehyde with half lives varying between 2.5 and 12 hr [8]. These compounds are rapidly converted to 3-phenoxybenzoic acid (3-PBA). The 3-PBA is conjugated to glucuronic acid that renders the xenobiotic more polar and facilitates its excretion in the urine [9].

Biomonitoring of pyrethroid exposure usually involves detecting the 3-PBA metabolite after acid hydrolysis of the urine [10, 11] but authors caution that urine should be collected within 24 hr after exposure and that 3-PBA excreted in the urine cannot be used to assess active dose [12]. Moreover, several studies have shown that there is no correlation between the concentration of the urinary metabolite and the symptoms mentioned [13, 14, 15]. This observation led us to seek alternative procedures for biomonitoring of exposure to pyrethroid insecticides. It is well known that protein adducts of xenobiotics represent a more persistent biomarker having half lives up to several months compared to urinary metabolites and in the case of chronic toxicity protein adducts were found in human tissues that have provided mechanistic insight into the epidemiological associations between smoking and cancer [16]. A previous study found that adverse health effects resulted from high dose acute exposure to parent compound (pyrethroid/pyrethin) and not from metabolites [17], but little is known about effects of chronic exposure.

Several studies have developed methods for detecting 3-PBA-adducts using mass spectrometric analysis [18, 19, 20, 21]. These studies are based on the analysis of chemical structure. The β-glucuronides of the two major carboxylic acid metabolites of pyrethroids i.e. permethrin, 3-PBA and *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (Cl_2CA), are electrophilic compounds that can form adducts with endogenous proteins [22]. Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished. According to the transacylation mechanism, nucleophilic sites in the proteins are bound by the O-acyl glucuronide and the UDP-glucuronic acid group is lost. Meanwhile in the glycation mechanism, an initial internal acyl migration occurs in O-acyl glucuronides, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts [23, 24], which may eventually undergo a (slow) Amadori rearrangement. The adduct formation of the β-glucuronides of the two permethrin metabolites, 3-PBA and Cl_2CA, with a model compound (i.e. albumin because it is the most abundant protein (60%) in human blood plasma) has been demonstrated [9].

Current analytical methods used for 3-PBA metabolites in plasma are based on multistep sample cleanup procedures including hydrolysis, extraction, and derivatization. Samples analyzed using high-performance liquid chromatography (HPLC) showed low sensitivity with limits of detection (LOD) ranging between 20 and 100 ng/mL, while limits of quantitation (LOQ) were 150–200 ng/mL [25]. The 3-PBA metabolites in cord blood were also determined in pregnant women because pesticides can act as neurotoxins affecting the fetus during the state of rapid growth and development of the brain [26]. These above analytical methods are time-consuming, expensive, are not suitable for routine analysis and are of low sensitivity. Immunoassay is an interesting method for sensitive and rapid

assessment to detect trace chemicals such as pesticides and their metabolites as key biomarkers of exposure [27].

Immunoassay is a biochemical technique using the specific binding of an antibody to its antigen. The quantity of antigen or antibody can be detected by labeling either the antigen or antibody. In this study, we analyzed by competitive enzyme immunoassays, the antigen in the sample competed for limited antibody binding sites with an antigen mimic conjugated to a carrier protein. The bound antibody was detected by reaction with a secondary antibody labeled with an enzyme. The resulting signal was inversely proportional to the antigen (3-PBA) concentration. An enzyme-linked immunosorbent assay (ELISA) is a suitable and easy application in the field. Moreover, the ELISA method provided higher sample throughput with lower cost as compared to the GC-MS method for permethrin [28].

We have developed an immunoassay for detecting an active biomarker to study human exposure to pyrethroid insecticides that measures total 3-PBA including the adduct form in plasma. The aim of this study was to develop an ELISA method including sample preparation for the analysis of 3-PBA in plasma using a polyclonal antibody. The developed method was applied to detect 3-PBA in real samples for testing the ability of the developed method for field application. The preparation of hapten and the production of antibody were reported previously [27]. This study reports assay optimization, and validation for plasma analysis.

EXPERIMENTAL

Materials and instrumentation

Bovine serum albumin (BSA), fetal bovine serum, goat anti-rabbit immunoglobulin G (whole molecule) peroxidase conjugate as the secondary antibody, H_2O_2, Tween 20, dimethyl sulfoxide, 3,3′,5,5′-tetramethylbenzidine, *N*,*N*-dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were reagent grade or better from Fisher Scientific (Pittsburgh, PA, USA) or Merck (Darmstadt, Germany). The samples were extracted using Strata Screen-A mixed mode solid phase extraction columns (8B-S019-EAK, Phenomenex, Torrance, CA, USA). The ELISA method was performed on Nunc Maxisorp Immunoplates (96F, 442404, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Tecan Sunrise, Männedorf, Switzerland).

Study population and sample collection

A consent form was introduced and signed by individual participants prior to collection of blood samples. The participants included 50 consumers and 50 farmers from Fang district, Chiang Mai province which is an intensive agricultural area in northern Thailand. Approximately 10 mL of blood sample was drawn by venous puncture into heparinized vacutainer tubes. Plasma was isolated for further analytical procedures. This study was approved by the Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University (N0.32/2006).

Plasma sample preparation

Alkaline hydrolysis—A 0.1 mL aliquot of 6N sodium hydroxide (NaOH) was added to 0.5 mL of homogenized plasma and placed in a heating block at 100 °C for 1 hr.

Liquid-liquid extraction (LLE)—1 mL of 0.2 M sodium acetate buffer, pH 4.5 was added to adjust the pH to around 12 to obtain the ionic form (hydrophilic) of 3-PBA. For the

cleanup step, 2 mL of ethyl acetate was added to the sample. The ethyl acetate removes organic contaminants while the 3-PBA remains in the aqueous phase. After shaking vigorously for 10 min and centrifugation, the aqueous phase (lower phase) was collected. The remaining aqueous phase was acidified (pH around 3) to produce the nonionic form of 3-PBA (hydrophobic) by adding 120 μL of 6N hydrochloric acid (HCl). It was extracted twice with 2 mL of ethyl acetate. Following shaking and centrifugation, the organic phase (upper phase) was combined and evaporated to dryness with a gentle stream of nitrogen. The residue was dissolved in 200 μL of methanol. Two milliliters of 0.2 M sodium acetate buffer was added to adjust pH to around 5 and mixed thoroughly.

Solid phase extraction (SPE)—The SPE method was modified from a previously reported method [29] using mixed-mode solid phase extraction (C8+anion exchange). The SPE cartridge used was the Strata screen-A (55 μm, 70 Å), 100 mg / 1 mL containing the mixed mode silica-based sorbent. The mixed-mode SPE hydrophobic (C8) and ionic (anion exchange) phases are shown in Fig. 2. A set of SPE tubes was placed into the vacuum manifold. The liquid was allowed to flow through under gentle vacuum at a flow rate of about 2–3 mL/min for each addition of liquid to the tube. The mixed mode cartridge was preconditioned with 1 mL of methanol, 1 mL of water and 1 mL of 0.2 M sodium acetate buffer (pH 4.5). The extracted plasma was loaded onto the cartridge and subsequently washed with 1 mL of water and 1 mL of methanol, sequentially to reduce matrix effects. The cartridge was dried under high vacuum (10 mmHg) for 5 min. After the test tube was replaced, the 3-PBA was eluted with 1.5 mL of 1% acetic acid in a mixture of hexane and ethyl acetate (70:30 v/v). The eluate was dried under nitrogen. The residue was dissolved in methanol (0.25 mL) followed by addition of 2.25 mL of phosphate buffered saline (PBS) to make a 5 fold dilution based on the original plasma amount (500 μL) prior to ELISA analysis.

Immunoreagents

The specific antibody and hapten for the target analyte (3-PBA) were previously described [27]. Briefly, two New Zealand white rabbits were immunized with 3-[4-(3-carboxyphenoxy) phenoxy] N-thyroglobulin ethylamine. The antigen solution, 100 μg in PBS, were emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. After 1 month, the animals were boosted with additional immunogen (100 μg) that was emulsified with Freund's incomplete adjuvant (1:1 v/v). Booster injections were given at 4 week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters. This terminal bleed of rabbit no. 294 was used for ELISA development.

Competitive indirect enzyme-linked immunosorbent assay

The preparation of coating antigen [30], the buffers and the procedure for the indirect competitive ELISA was previously described [27]. Briefly, 96-well plates were coated overnight at 4°C with 100 μL of coating antigen 3-PBA-BSA (0.5 μg/ mL) in 0.05M sodium carbonate-bicarbonate buffer, pH 9.6. The following day at room temperature, the coating antigen was washed off the plate with a PBS solution containing 0.05% Tween 20 (PBST), pH 7.5, and wells were blocked for 30 min with 200 μL 0.5% BSA in PBS. The ELISA plate was washed 5 times and 50 μL of plasma extract and 50 μL of antiserum (1:7000 in PBST) were added to each well and mixed on a plate shaker for 1 hr at room temperature. After washing 5 times, 100 μL of the goat anti-rabbit IgG-horseradish peroxidase conjugate (GAR-HRP) diluted 1/10000 in PBST was added into each well and the plate incubated for 1 hr at room temperature. Following a wash, a colorless substrate (100 μL) was added to each well and incubated for 15 min at room temperature in the dark.

The substrate solution contained 0.4 mL of 0.6% of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) in dimethyl sulfoxide and 0.1 mL of 1% hydrogen peroxide (H_2O_2) in 25 mL of citrate-acetate buffer, pH 5.5. Fifty microliters of 2N H_2SO_4 was added as a stop solution. Absorbances were obtained at a wavelength of 450 nm. The concentration of 3-PBA in the unknown samples was calculated based on the calibration curve.

Quality assurance and control

For laboratory quality assurance and control, precision measurements were conducted prior to the analysis of real samples. Pooled plasma controls (10 tubes) were analyzed for intra-assay variation (within a single day) of samples to obtain the OCV range (mean ± 1SD). Each batch of samples analyzed (n = 20) was composed of 1 sample blank and 2 controls that were added in the first and last tubes. For inter-assay variation samples were analyzed on different days.

Statistical analysis

Data from laboratory results of the pilot study were analyzed by SPSS Statistical Software Package version 17 as follows; (1) the concentration of 3-PBA in plasma was tested for normal distribution (parametric or nonparametric), (2) % detection, median and min-max of 3-PBA concentration were computed.

High-performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOFMS)

High Resolution Mass spectrometry—All the exact mass measurement experiments were performed in positive mode, on Micromass LCT an orthogonal acceleration-Time-of-Flight (oa-TOF) mass spectrometer (Micromass, Manchester, UK) configured with dual sprayer electrospray ion source, a standard Z-spray electrospray (ES) ionization source and a Lock-spray source that samples analyte and reference ions independently. The mass spectrometer has been equipped with a 4 GHz time-to-digital converter (TDC).

Ion source conditions—Ion source parameters were: capillary voltage 3200 V, sample cone voltage 35 V, extraction cone voltage 5 V, source temperature 110 °C and desolvation temperature 300 °C. *Transfer optics* settings were follows: rf lens 375 V, rf dc offset-1, 6.0 V, rf dc offset-2, 4.0 V aperture, 4.0 V, acceleration 200.0 V, focus 0.0 V and steering, −1.0 V. *Analyzer settings* were as follows: MCP (multi channel plate) detector 2700 V, ion energy 40.0 V, tube lens, −1.0 V, grid-2 5.0 V, TOF flight tube 4578 V and reflectron 1813 V. The pusher cycle time was 75 μs, data files were acquired in continuum mode and spectra were stored from m/z 100 to 2100 with a 2.1 second scanning cycle consisted of a 2.0 second scan and a 0.1 second inter-scan time. Each averaged spectrum stored to the data system, represented an average of 2,000 individual spectra. Typically 20–30 individual spectra were summarized. The cone gas and desolvation gas was set to 12 and 730 L/hour, respectively. Lock spray parameters were identical to sample setting parameters. Lock spray sampling frequency mode was set to 4, i.e. every 4th spectrum generated was the signal from the lock spray inlet. Mass resolution was set to 6000.

TOF Calibration and Lock-Mass set up—Lteff (effective length of the flight tube) value has been set to 1125.0700 using molecular ions of Leucine Enkephalin at 556.2771 Th. This value is responsible to set the zero cut on the calibration (error) function (5th order polynomial). System calibration was performed using Poly-D-L-alanine (P9003, Sigma, MO, USA) in both, positive and negative mode; a 50 ng/mL solution of Leucine Enkephalin (L9133, Sigma, MO, USA) has been infused at 5 μL/min into the lock spray (positive ion lock mass: 556.2771 Th) using ISCO μLC-500 micro flow pump. To obtain accurate masses the following procedure was performed: Savitsky-Golay smoothing using ± 4 channel

window, repeated twice and centering, using the center value at the 80% height of the peak. After data acquisition and signal averaging, spectra were individually corrected relative to the Leucine Enkephalin lock-mass calibration mass via standard procedure. Samples were introduced to the mass spectrometer via direct flow injection using Waters Alliance 2795 (Bedford, MA, USA) HPLC system was used for solvent delivery at the flow rate of 25 μl/min, mobile phase ACN/H_2O (1/1) was used.

HPLC Separation—Waters Alliance 2795 (Bedford, MA, USA) HPLC system was used for solvent delivery at the flow rate of 0.35 mL/min.

Mobile phase, gradient—Solvents - A: D.I. water 99.9, and Trifluoroacetic Acid (Thermo Scientific, Rockford, Il, USA), 0.1 volume %, B: ACN (Fisher Scientific, USA) 99.9, Trifluoroacetic Acid 0.1 volume %. Gradient: 0 –5 min 20 % B, 105 min 80% B, 110 min 100 % B, 116 min 100 % B, 117 min 20 % B, 120 min 20 % B. Sample temperature was kept on 20 °C.

Column—For separation Waters Symmetry300, C18, 250X4.6 mm, 5 μm HPLC column was used. Column temperature was kept on 20 °C. Instrument back pressure has not exceeded 150 bar.

UV-VIS detector—For UV-VIS signal detection Waters 996 PDA detector was used, wavelength range 210–550 nm, resolution 1.2 nm, with sampling rate of 1 spectrum/s.

Data processing—Data acquisition, instrument control was performed by MassLynx software version 4.0 sp 3, also used for data evaluation and visualization.

RESULTS AND DISCUSSION

Optimization of the ELISA method

The previous report described suitable conditions for the analysis of urinary 3-PBA [27]. This study is the first to detect 3-PBA in plasma using an ELISA method. ELISA inhibition curves were prepared in fetal bovine serum after alkaline hydrolysis, LLE, and SPE. The FBS was then spiked with 3-PBA (0, 1, 2.5, 5, 10, 20, 40, 80, 160, 320, 1280, 2560 and 5120 ng/mL; all concentrations were run in duplicate). The optimized ELISA using antiserum (rabbit no. 294), 1/7,000 (final dilution in wells); coating antigen 3-PBA-BSA, 0.5 μg/mL gave an IC_{50} value of 26.7 ng/mL. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation, $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum signal at no analyte, B is the curve slope at the inflection point, C is the IC50, and D is the minimum signal at infinite concentration. The IC50 value was expressed as the sensitivity of the immunoassay an approximates the concentration of analyte giving 50% inhibition (Fig. 2).

Validation of the method

The LLE extraction was necessary in this study to remove interferences and increase %recovery before SPE and ELISA analysis because plasma contains many components. It is mostly water (93%) and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones, carbon dioxide and excretory products [31]. For further cleanup, 1 mL of 0.5 N NaOH was added to get ionize the 3-PBA (hydrophilic) so the target analyte was extracted into the aqueous phase. After centrifugation, the organic phase was discarded. The remaining aqueous phase was acidified by adding ten drops of 6 N hydrochloric acid to obtain the nonionic form of 3-PBA (hydrophobic) and was extracted, twice, with 1 mL of

ethyl acetate. This method was found satisfactory to eliminate the matrix effect of samples before SPE and ELISA analysis because the results showed high recovery values (85.9–99.4%) in Table 1. In addition, this method also effectively removed the plasma matrix and allowed the ELISA to quantitate low parts per billion levels of 3-PBA in plasma (5 ng/mL). Table 1 showed that this assay was 30- to 40-fold more sensitive than a previous study (150–200 ng/mL) that used HPLC analysis [25]. The LOQ, limit of quantitation, is the lowest concentration that can be reliably detected in a sample or gave recovery more than 80% in spiked samples. The spiked samples with levels lower than 5 ng/mL i.e. 1, 2, 3, 4 ng/mL showed % recoveries of 135.6, 115.0, 128.4, and 111.3, respectively. This higher recovery may be due to matrix effects when analyzing very low concentrations. Moreover, the standard curve showed little change in %B/B0 between 0–2.5 ng/mL. The most accurate results are obtained where the %B/B0 is in the linear portion of the calibration curve. Therefore, samples with concentrations lower than 5 ng/mL were not quantitated.

The 3-PBA measured in this study is the total including free 3-PBA and the 3-PBA-adduct. It is difficult to separate the 3-PBA-adduct from free-3-PBA because the concentration of 3-PBA in plasma is very low (parts per billion). Moreover, it has been reported that humans exposed to cyfluthrin eliminated free 3-PBA from plasma with a very short half life of about 6 hour after oral or inhalation exposure. The 3-PBA was mostly eliminated in urine (93%) during the first 24 hour [12]. 3-PBA-adducts have been shown to have half lives up to several months [16].

In Table 3, the recovery of free 3-PBA in plasma was nearly 100% (95.6% in day 1 and 102.2% in day 2) demonstrating a high efficiency and reproducibility for this method. Moreover, the developed method could separate more than 80% 3-PBA from the adducted form (3-PBA-BSA) with almost the same %recovery as spiked free 3-PBA. For testing this hypothesis, we used BSA conjugated to 3-PBA for analysis because albumin is the most abundant protein (60%) in human blood plasma [32]. The 3-PBA-BSA was synthesized [33] and the ratio of 3-PBA to BSA was analyzed by high-performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOFMS). The chromatographic peaks showed different retention times because different positions of the reactant amino acid residues (e.g. lysine, cysteine, histidine) result in different physical characteristics of the molecules. We used the lowest molar ratio of 3-PBA to BSA (22, Table 2) in order to estimate the highest %recovery of 3-PBA-BSA. The expected % recovery range should be 60.9–100.2 % calculating from 47 and 22 molar ratios of 3-PBA to BSA. We then calculated the amount of 3-PBA-BSA that would yield 10 ng/mL of 3-PBA following hydrolysis. This amount of 3-PBA-BSA was spiked into fetal bovine serum before alkaline hydrolysis, LLE, SPE and ELISA analysis. Separate samples were spiked with free 3-PBA at 10 ng/mL. The recovery results are shown in Table 3.

Quality Assurance and Control

The coefficient of variation (%CV) of controls (pooled plasma) in each batch (2 tubes; intra-variation) and %CV of control concentration in all batches (10 values; inter-variation) were less than 5% and these 3-PBA concentrations were within the OCV range (Mean±1SD; 6.05–6.57 ng/mL) as shown in Table 4.

Application of the method

The developed method was successfully applied to detect 3-PBA in real samples between consumers (n=50) and farmers (n=50) with no matrix effect, shown in Table 5. The results showed that farmer group (40% detection) had an accumulated dose of pyrethroids (3-PBA-adduct) greater than the consumer group (22% detection) and also the median value of 3-PBA in farmer group (6.27 ng/mL) was greater than the consumer group (5.82 ng/mL).

Presumably this is because farmers were exposed to these insecticides from several routes such as usage in household products for controlling vectors, usage of national program for controlling mosquito vectors [1], consumption of pesticide residues in vegetables and fruits and occupational activities i.e. spraying of pesticides in agricultural areas [2].

This assay could analyze 20 samples per day and the ELISA method generated data within 3 hours that was rapid and suitable for routine analysis. Each of these tests cost $6.00 per sample, compared to the relatively high cost ($50–150 per sample) of instrumental analysis such as LC-MS/MS. To our knowledge, this is the first ELISA method for detecting 3-PBA in human plasma and applied to field samples.

CONCLUSIONS

A sensitive and specific ELISA has been developed with an IC_{50} value is 26.7 ng/mL. The developed ELISA coupled to LLE and SPE for sample preparation was successfully applied to detect 3-PBA in real samples with an LOQ of 5 ng/mL. Moreover, plasma 3-PBA as an active biomarker was also detected using this methodology. We proposed that this method is useful for pyrethroid exposure assessment among farmers and consumers in agricultural areas. It provides a means for chronic toxicity assessment that may allow determination of the epidemiological associations between endocrine disorders related disease and the 3-PBA biomarker.

Acknowledgments

This study was funded by grants from the Royal Golden Jubilee Ph.D. program, the Thailand Research Fund (PHD/0184/2547), the United States National Institute of Environmental Health Science P42ES04699 and the Center for Disease Control/National Institute of Occupational Health Science U50 OH07550. ST would like to deeply thank the Research Institute for Health Sciences, Chiang Mai University for the great support for this study. This study was also supported by the National Research University (NRU) Fund, Chiang Mai University.

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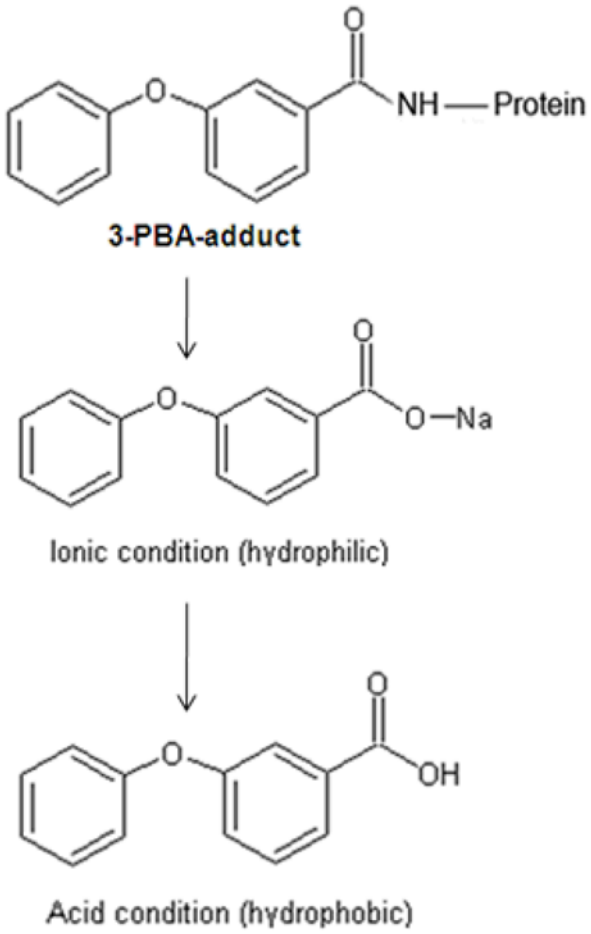

Fig. 1.
Forms of 3-PBA in the plasma sample during alkaline hydrolysis and the LLE method

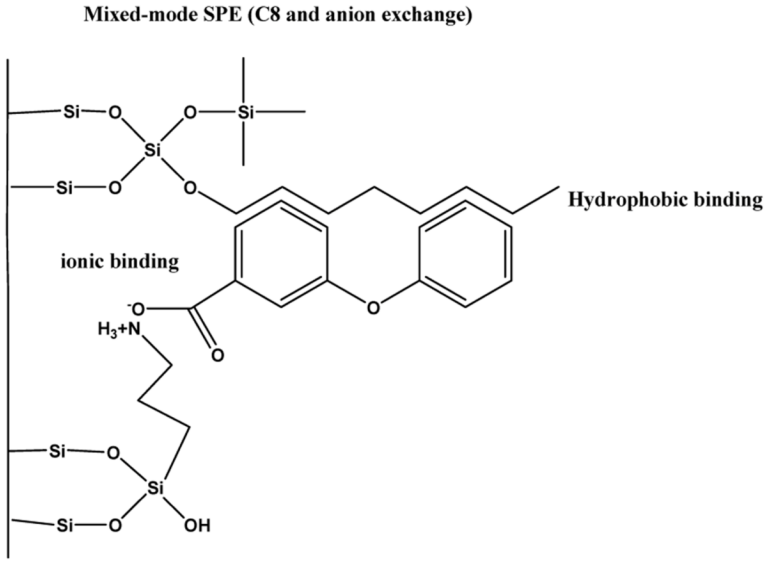

Fig. 2.
The target molecule (3-PBA) linked to dual mode SPE (C8 and anionic exchange) cartridge

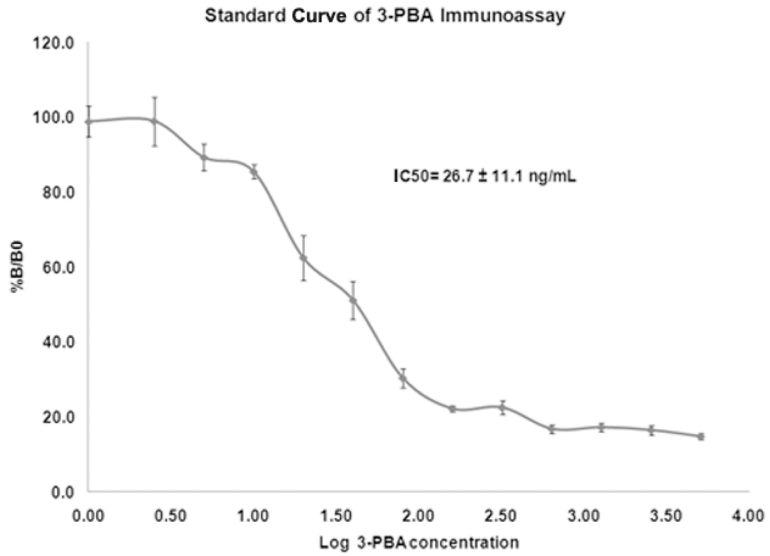

Fig. 3.

ELISA inhibition curve for 3-PBA using antiserum 294 (diluted 1:7,000, final dilution in well), coating antigen 3-PBA-BSA (0.5 μg/mL), and GAR-HRP (1:10,000). IC50, 26.7 ± 11.1 ng/mL, was the concentration of analyte giving 50% inhibition. The X axis is the log of the 3-PBA concentration and the Y axis is %B/B0 calculated from (absorbance of sample / absorbance of blank) X 100.

Table 1

Recoveries after hydrolysis, LLE, SPE and ELISA analysis of 3-PBA spiked in fetal bovine serum, in triplicate (n=3) on the same day.

Standard addition(ng/mL)	Mean ± SD concentration(n=3) of 3-PBA (ng/mL)	% Recovery	%CV
4	4.45±0.54	111.3	12.2
5 (LOQ)	4.97 ± 0.292	99.4	5.88
7.5	6.63 ± 0.677	88.4	10.2
10	8.85 ± 0.703	88.5	7.94
12.5	12.1 ± 0.921	96.5	7.64
15	12.9 ± 0.960	85.9	7.45

LOQ, limit of quantitation, the lowest concentration that can be reliably detected in samples was empirically set to 5 ng/mL.

Table 2

Retention time (RT), found mass, mass increment and number of 3-PBA to BSA for characterization of 3-PBA-BSA

Peak	RT (min)	Found mass (Da)	Mass increment	Number of 3-PBA to BSA
1	48.73	70723	4290	22
2	50.29	70949	4516	23
3	53.93	73091	6658	34
4	55.97	73509	7076	36
5	60.35	74016	7583	39
6	61.56	75655	9222	47

Molecular mass for calculation in this study of BSA and 3-PBA are 66,433Da and 196Da, respectively

Table 3

Recoveries after hydrolysis, LLE, SPE and ELISA analysis of spiked free-3-PBA and 3-PBA-BSA (10 ng/mL) in fetal bovine serum, done in duplicate in two days.

Spiked 10 ng/mL	Free-3-PBA		3-PBA-BSA	
	Day 1	Day 2	Day 1	Day 2
Mean ± SD (n=2)	9.6±0.88	10.2±0.15	9.9±0.06	8.0±0.06
%recovery	95.6%	102.2%	98.9%	80.4%

Table 4

The intra and inter batches of coefficients of variation (%CV) in controls (pooled plasma) for detecting 3-PBA in consumers (n =50) and farmers (n=50), in each batch of samples (n = 20)

Day	First--tube concentration (ng/mL)	Last-tube concentration (ng/mL)	%CV of controls (2 tubes; intra-variation)
1	6.22	6.49	3.03
2	6.47	6.47	0.00
3	6.37	6.37	0.01
4	6.29	6.48	2.21
5	6.34	6.14	2.25

%CV of controls (10 values; inter-variation) = 1.90

Table 5

Descriptive analytical data for 3-PBA detected in human plasma between consumers and farmers

Group	% Detection	Median (ng/mL)	Min-Max (ng/mL)
Consumers (n=50)	22	5.82	5.16–8.44
Farmers (n=50)	40	6.27	4.29–9.57