CHAPTERS 4

RESULTS AND DISCUSSION

4.1 Organophosphate and carbamate pesticide

4.1.1 Development of human salivary cholinesterase assay

The within-day %CV from the intra-assay and the between-day %CV from the inter-assay for SAChE and SBChE, RAChE and PBChE were 0.18.4.54% and 3.38%, 0.97.4.77% and 3.38%, 0.43. 4.88% and 2.24%, and 0.35.4.96% and 3.32%, respectively. Moreover, all mean activities of controls in all batches (n = 20) within the OCV range (mean ± 2SD: 3.19-4.00 mU /mL, 3.10-3.81 mU/mL, 5.11-5.80 U/mL and 3.73-4.28 U/mL, respectively) as shown in Table 4.1. The LOB, LOD and LOQ are shown in Table 4.2. The activities of all ChE in the present study were higher than the LOQ.

4.1.2 Application of developed methods to detect activities of BChE and AChE in saliva

In consumers, the medians of SAChE, SBChE, RAChE and PBChE levels were 4.03 mU/mL, 4.17 mU/mL, 4.18 U/mL and 4.28 U/mL, respectively, while the medians in farmers were 3.94 mU/mL, 4.25 mU/mL, 3.95 U/mL and 4.16 U/mL, respectively.

Table 4.1 The within-day %CV for pooled saliva and blood in each batch (3 controls; intra-assay) and the between-day %CV of control concentrations in all batches (60 controls; intra-assay) for 200 samples

Batches	SAChE	SBChE	RAChE	PBChE	The within-day %CV (3 controls; intra-assay)
1	3.97	1.65	4.16	2.56	
2	0.18	1.07	1.23	2.30	
3	4.11	4.59	4.15	2.04	
4	3.67	0.97	1.07	1.75	
5	4.49	2.30	4.37	1.63	
6	4.54	4.02	4.44	0.85	
7	4.26	4.57	0.43	1.57	
8	1.73	2.85	4.41	0.66	
9	4.19	1.85	2.52	1.76	
10	3.21	3.97	2.01	4.96	
11	4.02	3.36	2.15	0.35	
12	2.51	2.03	2.22	1.73	
13	2.82	3.35	1.88	1.23	
14	3.45	4.77	4.88	2.36	
15	1.10	2.03	3.04	1.93	
16	2.52	4.35	1.20	1.55	

Table 4.1 (continued) The within-day %CV for pooled saliva and blood in each batch (3 controls; intra-assay) and the between-day %CV of control concentrations in all batches (60 controls; intra-assay) for 200 samples

Batches	SAChE	SBChE	RAChE	PBChE	The within-day %CV (3 controls; intra-assay)
17	4.00	2.72	1.20	1.00	
18	1.71	1.16	2.31	1.98	
19	1.32	2.23	1.02	1.40	
20	3.83	4.30	2.07	2.13	
Total	3.38	3.38	2.24	3.32	
The between-day %CV (60 controls; inter-assay)					

Table 4.2 Limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) of methods for measurement of ChE activities

	Saliva (mU/mL)		Blood (U/mL)	
Value	AChE	BChE	AChE	BChE
LOB	0.660	0.489	0.784	0.251
LOD and LOQ	0.999	0.784	1.071	0.479

Moreover, previous research showed that SAChE was not feasible as a biomarker because the relatively low level of SAChE that was ~1000 times lower than RAChE levels of ~10–30% in saliva may well result in a high variability in low level detection (Yamalik *et al.*, 1990; Ng *et al.*, 2009). Although SBChE levels are significantly lower

(approximately 1000 times) than PBChE levels, PBChE showed variability of the activity of enzymes due to the disease status, as shown in Table 4.3.

The present study shows increased PBChE activity among volunteers who had hypertension (r = 0.198, P = 0.005, n = 26), hyperlipidemia (r =0.241, P = 0.001, n = 11) which is supported by a previous study (Alcantara *et al.*, 2002) and diabetes (r = 0.217, P =0.002, n = 8) which is supported by a previous study (Annapurna *et al.*, 1991). The patients with these diseases had increased PBChE activity due to the enzyme involved in the degradation of lipid/lipoprotein metabolism (Shirai *et al.*, 1988).

Females who take the contraceptive pill (r = -0.171, P = 0.015, n = 25) also showed decreased PBChE activity, which is supported by a previous study (Lepage *et al.*, 1985). The contraceptive pill that contained estrogen could reduce the hepatic synthesis and the release of PBChE (Sidell and Kaminskis, 1975). Therefore, SBChE compared with PBChE levels could be a suitable tool as a biomarker for pesticide exposure because the SBChE didn't show any variability due to the disease status on the activity of the enzyme.

4.1.3 Examination of the relationship between activities of BChE and AChE in saliva and blood

In consumers who had less exposure to pesticides there was no variability in low level detection of SBChE because the present study found a correlation (r = 0.232, P = 0.020) between the activity of SBChE and PBChE only in consumers (Figure 4.1).

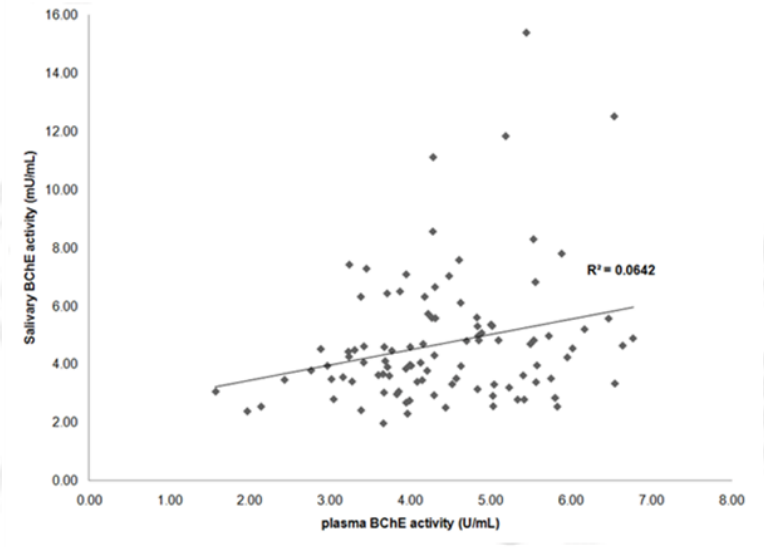

Figure 4.1 Bivariate scattergram of SBChE activities (mU/mL) and PBChE activities (U/mL) found correlation (r = 0.232, P = 0.020) between activities of SBChE and PBChE in consumers.

Results from the present study suggest that SBChE may be an alternative biomarker for pesticide exposure besides PBChE in consumers who generally have less exposure to pesticides than farmers. There is a limitation of the SChE inhibition test among low ChE activity like farmers with higher ChE activity inhibition than consumers. Farmers are generally highly exposed to commonly used ChE-inhibiting pesticides, and their ChEs are subsequently inhibited.

Therefore, farmers who were highly exposed to pesticides might have a higher variability at low levels of detection of SChE compared to consumers, and we didn't find any correlation of ChE activities between saliva and blood in farmers. Moreover, consumers are a lager population than farmers in Thailand, so that this method is suitable for application in most communities. A previous study showed that in total, the Thai population (63, 878, 267 people) contained only 1.02% farmers (652,209 people) (The

department of provincial administration comparison of ratio between total population and consumer group in each province in Thailand, 2010).

The present study shows that SBChE didn't have any variability due to the disease status'effect on the activity of enzymes, i. e. hypertension, hyperlipidemia, diabetes and taking the contraceptive pill. Moreover, in consumers who were less exposed to pesticides didn't have any variability in low level detection of SBChE because the present study showed a correlation ($r = 0.232$, $P = 0.020$) between the activity of SBChE and PBChE only in consumers.

Results from the present study suggest that SBChE may be an alternative biomarker for pesticide exposure beside PBChE among non-farmers or consumers. The modified methods in the present study showed sufficient sensitivity with a low LOQ and high accuracy when applied to measure ChE activity in saliva with low variability (%CV) i. e. by the intra-assay for within-day for %CV, and between-day for all enzymes.

However, a more sensitive method than the present developed method one should be developed to measure the relatively low activities of SAChE and SBChE among ChE inhibited farmers.

4.2 Synthetic pyrethroid pesticides

4.2.1 Development of sample preparation step and sensitivity ELISA method

4.2.1.1 Sample preparation

The methods reported here are selective and sensitive enough to assess samples from both acute and chronic exposures to pyrethroids. For example the RfD for pyrethroids is 0.25 mg/kg/day (Reregistration Eligibility Decision (RED) for Permethrin, 2007).

Table 4.3 Factors affecting variability from the status of diseases to activity of all ChE

Status of diseases	Median (n = 200)			
	Saliva (mU/mL)		Blood (U/mL)	
	SAChE	SBChE	RAChE	PBChE
Hypertension				
No (n = 174)	4.11	4.27	3.95	4.16
Yes (n = 26)	3.52	3.94	4.65	4.74 *
Hyperlipidemia				
No (n = 189)	4.01	4.22	3.94	4.17
Yes (n = 11)	3.93	4.63	4.91	5.54 *
Diabetes				
No (n = 192)	4.02	4.22	3.95	4.18
Yes (n = 8)	3.28	4.47	5.16	5.50 *
Contraceptive pill taking				
No (n = 175)	4.04	4.26	4.24	4.29
Yes (n = 25)	3.94	4.22	2.95	3.82 *

(*) = Found the difference between groups at the 0.05 level of significance using 2-Independent-Samples-Tests (Mann-Whitney U)

Assuming an average person weighs 70 kg, has a blood volume of 5 L, and consumes, in a single day, 0.25 mg of pyrethroid and all was absorbed and circulating in the blood, then the expected concentration in the blood would be about 3.5 µg/mL, well above the LOQs reported here. The 3-PBA in plasma 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 (Leng *et al.*, 1997). 3-PBA-adducts have been shown to have half lives up to several months (Phillips, 2002).

In Table 4.4, the recovery of free 3-PBA in plasma was nearly 100% (95.6% in day1 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, BSA conjugated to 3-PBA was used for analysis because albumin is the most abundant protein (60%) in human blood plasma (Presle *et al.*, 1996). The 3-PBA-BSA was synthesized (Shan *et al.*, 1999) and the ratio of 3-PBA to BSA was analyzed by 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. The lowest molar ratio of 3-PBA to BSA was used (22), Table 4.5 in order to estimate the highest % recovery of 3-PBA-BSA. The expected % recovery range should be 60.9-100.2 % calculating from 47 (the highest) and 22 (the lowest) molar ratios of 3-PBA to BSA. Then the amount of 3-PBA-BSA was calculated 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 4.4.

Table 4.4 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 4.5 Retention time (RT), found mass, mass increment and number of 3-PBA to BSA for characterization of 3-PBA-BSA (Molecular mass for calculation in this study of BSA and 3-PBA are 66,433Da and 196Da, respectively)

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

The 3-PBA immunoassay has been assessed for matrix effects from plasma. Recovery of a laboratory-generated 3-PBA-protein adduct (the 3-PBA was adducted to bovine serum albumin) spiked into fetal bovine serum were greater than 80%. In addition, we found that an additional LLE cleanup was necessary before SPE extraction to eliminate the matrix effect of plasma samples. This resulted in high recovery values

(85.9-99.4%) with a limit of quantitation (LOQ) at 5 ng/mL. For urinary 3-PBA, SPE extraction was necessary to determine 3-PBA metabolites. The SPE cleanup technique gave high recovery values (87.3-98.0%) with limit of quantitation at 2.5 ng/mL (Table 4.6).

Table 4.6 Recoveries after hydrolysis, extraction and ELISA analysis of 3-PBA spiked in fetal bovine serum and urine containing no detectable 3-PBA by ELISA in duplicate (n=2) on the same day.

Standard addition (ng/mL)		Mean ± SD concentration (n=3) of 3-PBA (ng/mL)		% Recovery	
plasma 3-PBA	urine 3-PBA	plasma 3-PBA	urine 3-PBA	plasma 3-PBA	urine 3-PBA
4	1	4.45±0.54	ND	111.3	ND
5	2.5	4.97 ± 0.292	2.32± 0.195	99.4	92.8
7.5	5	6.63 ± 0.677	4.47 ± 0.715	88.4	89.5
10	10	8.85 ± 0.703	8.73 ± 0.160	88.5	87.3
12.5	20	12.1 ± 0.921	19.6 ± 0.339	96.5	98.0

ND=Not detected, because the signal was not distinguishable from background.

The limit of blank, limit of detection and limit of quantitation as defined by Armbruster and Pry (2008) are shown in Table 4.7. The urine spiked with levels lower than the limit of quantitation were not detected (ND, Table 4.6) as expected. Analysis of 3-PBA spiked into fetal bovine serum at 4 ng/mL gave values nearly identical to 5

ng/mL. This higher recovery in serum may be due to a matrix effect hence the LOQ was set to 5 ng/mL. Samples with concentrations lower than 5 and 2.5 ng/mL, in serum and urine respectively, were not quantitated.

Table 4.7 Limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) of methods for measurement of plasma 3-PBA and urinary 3-PBA

Value	Plasma 3-PBA Mean± SD, n=2, (ng/mL)	Urinary 3-PBA Mean± SD, n=2 (µg/g Creatinine)
LOB	0.50 ± 0.03	0.46 ± 0.01
LOD	1.08 ± 0.20	1.94 ± 0.34
LOQ	5.00 ± 0.19	2.50 ± 0.37

4.2.1.2 Optimization of the ELISA method

The assay for 3-PBA in urine was also previously reported. The IC_{50} value in that study was reported at 1.65 ± 0.7 ng/mL for urine 3-PBA (Shan *et al.*, 2004). In our hands, the optimized assay using antiserum 294 (diluted 1 : 7000, final dilution in well) and a coating antigen concentration of 0.5 µg/mL gave an IC_{50} value of 15.3 ± 0.77 ng/mL (urine 3-PBA) and 26.7 ± 11.1 ng/ml (plasma 3-PBA) in Figure 4.2. Although the IC_{50} was different, it was highly reproducible, so no further optimization was done. It is not unusual for them to be systematic differences in IC_{50} between laboratories. Minor differences between operations, preparation of calibrators, or reagent degradation are among some of the causes of such variations (Herman and Shan, 2011).

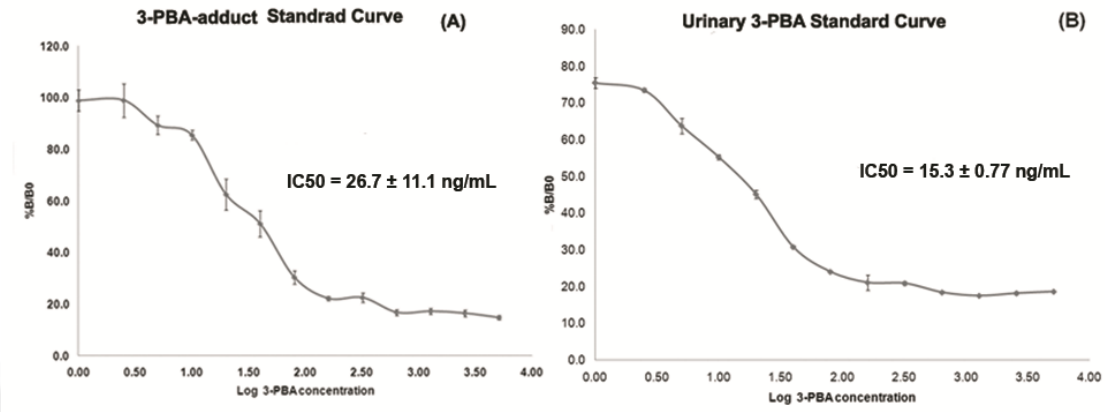

Figure 4.2 ELISA inhibition curve for plasma 3-PBA (A) and urine 3-PBA (B) using antiserum 294 (diluted 1 : 7,000, final dilution in well), coating antigen 3-PBA-BSA (0.5 µg/mL), and GAR-HRP (1 : 10,000)

Most immunoassays exhibit some cross reactivity to structurally related compounds. The assay used here does not cross react with a variety of parent pyrethroids (permethrin, cypermethrin, esfenvalerate, deltamethrin and cyfluthrin) that contain the phenoxybenzyl moiety. However, 4-fluoro-3-phenoxybenzoic acid, a metabolite of cyfluthrin does cross react at 72% (Shan *et al.*, 2004) while 4-hydroxy-3-phenoxybenzoic acid and 3-phenoxybenzaldehyde cross react at 103 and 75%, respectively.

Using the same methods as reported here, urine samples collected from forestry workers were analyzed by both LC/MS/MS and immunoassay for 3-PBA. The methods correlated well with slope, intercept and r^2 of 1.14, 0.24 and 0.964, respectively (Ahn *et al.*, 2011). Thus, these other cross-reacting metabolites likely did not greatly impact the determination of urinary 3-PBA for that study population and were not considered significant in the study reported here.

4.2.1.3 Quality assurance and control

For quality control, pooled samples of plasma or urine were used. Two or three replicates of the pooled samples were run with each batch of samples. The coefficient of variation (% CV) was less than 5% for both plasma and urine. Moreover, all control concentrations were within the OCV range (mean ± 2SD; 5.79 - 6.83 and 14.8 - 17.8 ng/mL, respectively) as shown in Table 4.8.

4.2.2 Application of developed methods to detect plasma 3-PBA

From questionnaire, data showed that the volunteers in this study used pesticides in the home and in agriculture practices. Of the total study population (consumers and farmers; n = 200), 56% used pesticides indoors. The concentration of plasma 3-PBA in the volunteers who used indoor pesticides (n = 106) was significantly higher ($p < 0.005$) than who did not use them (n = 94).

Twenty five percent of the farmers (n = 100) applied pesticides agriculturally. The farmers (n = 100) used a variety of pesticide types - 83% insecticides including synthetic pyrethroids, 75% herbicides, 72% chemical fertilizers, 56% organic fertilizers and 41% plant growth substances. The % detection of plasma 3-PBA was less than 50% overall while the detection of urinary 3-PBA was greater than 50% for both groups (Table 4.9).

Although the rates of detection in plasma or urine were similar between the groups, the range of urinary concentrations was generally higher for the farmer group and the median concentrations were significantly different a $p < 0.01$ likely reflecting recent acute exposures in the farmer group.

Table 4.8 Intra- and inter-assay variation of pooled controls for plasma and urine

Plasma 3-PBA				Urinary 3-PBA				
Batches	First-tube	Last-tube	%CV of controls	Batches	First-tube	Mid-tube	Last-tube	%CV of controls
	Concentration (ng/mL)		2 tubes; intra-variation		Concentration (ng/mL)			3 tubes; intra-variation
1	5.95	5.95	0.01	1	17.3	16.4	17.2	2.88
2	6.22	6.49	3.03	2	15.6	15.2	16.2	2.95
3	6.12	5.82	3.52	3	16.5	16.1	15.8	2.13
4	6.47	6.47	0.00	4	16.1	15.7	16.8	3.58
5	6.22	6.27	0.57	5	15.9	15.7	16.9	4.08
6	6.66	6.27	4.34	6	16.1	16.4	15.7	1.90
7	6.37	6.37	0.01	7	17.3	16.4	17.2	2.88
8	6.50	6.47	0.38	8	15.6	15.2	16.2	2.95
9	6.29	6.48	2.21	9	16.5	16.1	15.8	2.13
10	6.30	6.24	0.74	Plasma 3-PBA; %CV of controls (27 tubes; inter-variation) = 4.60				
11	6.15	6.09	0.75					
12	6.22	5.96	3.02	Urinary 3-PBA; %CV of controls (28 tubes; inter-variation) = 4.14				
13	6.34	6.14	2.25					
14	6.10	5.91	2.34					

Table 4.9 Descriptive analytical data for plasma 3-PBA and urinary 3-PBA detected in human plasma between consumers (n=100) and farmers (n=100)

Metabolites	% Detection		Median		Range	
	Consumer	Farmer	Consumer	Farmer	Consumer	Farmer
Plasma 3-PBA (ng/mL)	24	42	5.82	6.27	5.16 - 8.44	4.29 - 9.57
Urinary 3-PBA (µg/g creatinine)	76	69	8.86	16.1*	1.62 - 80.5	0.80 - 256.2

* Significantly different between groups at $p < 0.01$ using a paired T test.

Longer term exposure seems more similar between the groups since the median and range of plasma 3-PBA concentration was similar between groups. In Table 4.10, the % detection of plasma 3-PBA and urinary 3-PBA for consumer and farmer groups are broken out by concentration ranges. Plasma concentrations for all subjects were less than 10 ng/mL although the rate of detection was nearly double in farmers compared to consumers in the 5.1-10 ng/mL range. Similarly more farmers than consumers had urinary concentrations of 3-PBA greater than 10 µg/g creatinine and the upper range of concentrations were much higher for farmers. Additionally, the concentrations of urinary 3-PBA in farmers in Chiang Mai at selected percentile values in the present study were higher than found by previous researchers (Figure 4.3) (Panuwet *et al.*, 2004; Panuwet *et al.*, 2008). This may be due to different agricultural practices.

Thus, the farmer group appears to have greater acute exposure most likely due to their agricultural activities (Panuwet *et al.*, 2008). Consumers that lived in communities where crops were produced also likely had more potential for exposure to insecticides

(Schettgen *et al.*, 2002). However, there are other pathways and routes by which both farmers and consumers may be exposed to these insecticides. Consumption of pesticide contaminated vegetables and fruits was thought to be the major route by which volunteers were exposed to pesticides. The other routes were dermal contact and the respiratory route from insecticide drift in the air from vector control programs (The World Health Organization, 1995) and from usage in the household for controlling insects.

4.2.3 Examination the relationship between plasma 3-PBA and urine 3-PBA

Interestingly, there was no correlation between plasma 3-PBA and urinary 3-PBA concentrations in the study population. However, the farmer population likely had more recent acute exposures that would lead to transient high urinary 3-PBA concentrations. Also, metabolism and urinary elimination of pyrethroids varies by individual (Kuhn *et al.*, 1999). These results can explain according to the general metabolic pathways of pyrethroids such as permethrin in mammals presented in Figure 2.6 (Noort *et al.*, 2008).

After humans are exposed to pyrethroids, these compounds have been reported to be absorbed into serum and hydrolyzed by an esterase enzyme to 3-phenoxybenzyl alcohol or 3- phenoxybenzaldehyde. These compounds are rapidly converted to 3-phenoxy benzoic acid (3-PBA). The 3-PBA is conjugated to glucuronic acid that renders the xenobiotic more polar and facilitates its excretion in urine. The 3-PBA is a specific metabolite of some, but not all commercially available pyrethroids. Another metabolite is *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid that derive from parent compounds such as cyfluthrin, permethrin, and cypermethrin (Olsson *et al.*, 2004).

Table 4.10 The % detection of plasma 3-PBA and urinary 3-PBA in consumer and farmer groups by concentration ranges

Concentration ranges	% Detection			
	Consumer (n=100)		Farmer (n=100)	
	plasma 3-PBA (ng/mL)	urinary 3-PBA (µg/g creatinine)	plasma 3-PBA (ng/mL)	urinary 3-PBA (µg/g creatinine)
Less than 2.5	76	27	59	36
2.5-5.0	(Less than 5)	15	(Less than 5)	10
5.1-10.0	24	26	42	11
10.1-20.0	-	10	-	12
20.1-30.0	-	7	-	6
30.1-40.0	-	5	-	6
40.1-50.0	-	3	-	6
More than 50	-	7	-	13

The excess 3-PBA will bind to albumin in blood producing 3-PBA-adducts (Phillips, 2002). Two mechanisms of adduct formation from glucuronidation of carboxylic acids is shown in Figure 2.7 and 2.8 (Noort *et al.*, 2008). In the transacylation mechanism, acyl glucuronides that are potentially electrophilic can react with nucleophilic residues in proteins (Figure 2.7) (Noort *et al.*, 2008).

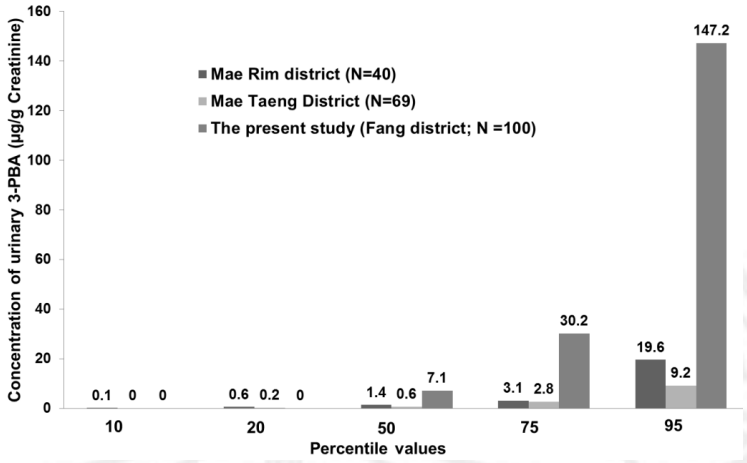

Figure 4.3 The concentrations of urinary 3-PBA in farmers in Chiang Mai province at Mae Rim district (N = 40; Panuwet *et al.* (2004) and Mae Taeng District (N = 69; Panuwet *et al.* (2008) at selected percentile values compared to the present study (Fang district).

According to the glycation mechanism, an initial internal acyl migration occurs and also reacts with amino groups of the protein; leading to Schiff base adducts (Figure 2.8) (Noort *et al.*, 2008) or Amadori rearrangement (Grubb *et al.*, 1993). The most likely protein for adduct of acyl glucuronides is human serum albumin (HSA) due to its abundance (60%) in human blood plasma (Presle *et al.*, 1996). However, further studies are necessary to determine the precise reasons for the difference.