A Pilot Survey of Pesticide-Specific Urinary Metabolites among Farmers in Chiang Mai Highland Agricultural Area

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ABSTRACT

This study surveyed the exposure to pesticides among farmers in Chiang Mai's highland agricultural area. Ethnic Hmong farmers, living in Baan Buak Jan, Mae Rim District, Chiang Mai Province were selected as the study population. Pesticide-specific urinary metabolites were used as biomarkers of exposure to a variety of pesticides, including organophosphorus pesticides, synthetic pyrethroids and selected herbicides. Our method employed a simple solid-phase extraction, followed by a gold standard analytical method, using isotope dilution high performance liquid chromatography- tandem mass spectrometry (LC/MS/MS). A total of 40 urine samples from Hmong farmers were analyzed for 19 specific pesticide metabolites. We found that para-nitrophenol (PNP, a specific metabolite of methyl parathion and parathion) was the dominant analyte, having the highest amounts in all urine samples tested. We also found the metabolites of chlorpyrifos, common pyrethroids and permethrin/cypermethrin, namely 3,5,6-trichloro-2-pyridinol (TPCY), 3-phenoxybenzoic acid (3-PBA) and cis- & trans-3-(2,2-dichlorovinyl)-2, 2-dimethylcyclopropane-1-carboxylic acids (c- & t-DCCA) respectively. The Hmong farmers were classified into groups according to their type of plantation or crop, resulting in the following breakdown: flower plantations (n=20), vegetable plantations (n=11) and flower and vegetable plantations (n=9). Thai farmers who had pesticide-free vegetable plantations (n=8) were used as a comparison group. The results showed that there was no significant difference among all analytes detected in farmers with different crop types (Mann-Whitney-U test $p>0.05$). However, a significant difference in PNP concentrations was found between farmers on plantations using pesticides and those on pesticide-free plantations (Mann-Whitney-U test $p<0.05$). No correlation was observed among individual analytes except for 3-PBA and t-DCCA (Pearson $p=0.00$), suggesting a common source for these two analytes, likely permethrin or cypermethrin insecticides. This study demonstrated pesticide exposure among farmers who use pesticides on their plantations in Thailand.

Further studies are required to determine if any pesticide-related health outcomes are associated with these exposures.

Key words: Urinary metabolites, Pesticides exposure, LC/MS/MS, Farmers in Thailand

INTRODUCTION

In Thailand, pesticides, particularly those in the organophosphate and carbamate groups, are widely used for agriculture and household purposes. Production of fruits and other high-value crops for export and local consumption often involves extensive use of agrochemicals, including pesticides (Kunstadter et al., 2001). As farmers have gradually switched from low-value to high-value crop production, the overall consumption of pesticides has naturally increased. In order to raise yield, farmers have intensified pesticide use in the production of all crops, as reflected by increasing shares of pesticide costs in total production costs (Paopongsakorn et al., 1998).

Thailand is a major market for pesticides which are mostly imported for industrial and agricultural use. Since pesticides were first imported into Thailand under the "Green Revolution Policy", part of the 1st National Economic and Social Development Plan in 1966, the total amount of imported pesticides has dramatically increased year by year. In 2000, the entire amount of imported pesticides was 52,739 tons which cost about 7,294 million baht. Most were herbicides, followed by insecticides, disease control agents, plant growth regulators and so on. Using the WHO hazard categories, 55.93% of imported insecticides fell into the Ia category (extremely hazardous) and the Ib category (highly hazardous) (Alternative Agricultural Network, 2003).

Because pesticides are often overused, Thai farmers' current use of pesticides is highly inefficient and has led to chemical poisoning. For instance, in order to save labor costs associated with spraying, farmers often mix pesticides themselves, creating a "cocktail" of several chemicals without considering their synergistic effects. In addition, farmers frequently increase the concentration of pesticides in the belief that increased intensities will lead to better protection (Poapongsakorn et al., 1998). During 2000-2001, the project carried out by Thailand's Food and Drug Administration had found that organophosphorus showed major problem among group of exposed people (Food and Drug Administration., 2001).

Chiang Mai Province, known as the capital city of Northern Thailand, has seen rapid development in education, industry and tourism. However, agriculture is still the major occupation in rural areas, and consequently, many resources have been used and new technologies adopted to improve the agricultural systems which is comprised of the production systems and supporting ancillary components. Most of the farmers have basically aimed at increasing their production in order to increase their income, and in some cases, just to survive. Plenty of pesticides, therefore, have been applied at almost every step in agricultural practice. Pesticides are commonly used among agriculturists and the rate of application in the agricultural sector is increasing every year (Nakma, 2002).

Recently, the research done among Hmong farmers in Chiang Mai Province, Thailand suggested that half of the studied group had risky or unsafe levels of cholinesterase

inhibition, an indicator of exposure to organophosphate and carbamate group of pesticides (Kunstadter et al., 2001). However, this result indicated only the exposure to organophosphate and carbamate group of pesticides. Yet, in order to monitor the exposure to various types of pesticides, fast and robust analytical methods, like liquid chromatography-tandem mass spectrometry (LC/MS/MS), need to be used for measuring the biomarker of pesticide exposure in urine samples.

MATERIALS AND METHODS

Study site and population

We have selected Baan Buak Jan, Mae Rim District, located 50 kilometers northwest of Chiang Mai City, as a study site. The entire population belongs to Hmong minority group of hill tribes. This village has approximately 200 households or about 700 people. Cash crop plantation such as cabbage, lettuce and flowers served as the major source of their income. From the household survey using individual interview and questionnaires, it was found that a lot of pesticides had intensively been applied over decades in order to improve both the quantity and quality of crop production. Most of the pesticide applicators are now at risk of high exposure to pesticides due to the amounts of applied pesticides and lacking of understanding on self-protection from exposure.

Sample collection

The research protocol, including the urine collection procedure was performed under an Ethical Clearance which was reviewed and approved by the Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University.

Farmers who had documented agricultural activity and used high amounts of pesticides in their field were randomly selected for participation. Morning urine samples were collected from 40 farmers (pesticide-use farmers). In addition, general and in-depth data from questionnaires were also collected among selected participants in order to gain information on pesticide application.

The sampling time for urine collection among the farmers was the morning, following application of pesticides in their field. Approximately 50 mL of morning urine was collected in plastic tubes and kept in a freezer at -20 0C prior to analysis.

Also 8 morning urine samples from farmers who performed pesticide-free plantation were collected as a comparison group.

Specific metabolite monitoring

We had selected 19 urinary markers. Seven of these represented organophosphorus insecticides. The markers were specific hydrolysis products, namely, chlorpyrifos, coumaphos, diazinon, isazophos, malathion, methyl parathion and pirimiphos or their methyl counterparts. Five metabolites were measured to evaluate exposure to synthetic pyrethroids, namely, cyfluthrin, permethrin, cypermethrin, deltametrin. Urinary markers of exposure to six herbicides were measured. Atrazine, alachlor, metolachlor and acetochlor were analyzed as their mercapturic acid conjugates and 2,4-D and 2,4,5-T as the parent herbicides. The markers for DEET exposure were also measured. Table 1 shows the list of metabolites and their parent compounds detected in this method.

Table 1. The metabolites and their parent compounds detected in this study.

Metabolites	Parent compounds	Metabolites	Parent compounds	Metabolites	Parent compounds
PNP	Methyl Parathion	ATZ	Atrazine	DEAMPY	Pirimiphos
TCPY	Chlorpyrifos	ACE	Acetochlor	CIT	Isazophos
3-PBA	Pyrethroids	MET	Metolachor	CMHC	Coumaphos
cis- and tran-DCCA	Cyfluthrin, Permethrin, Cypermetrin	2,4,5-T	2,4,5-T	MDA	Malathion
4-F-3-PBA	Cyfluthrin	2,4-D	2,4-D	IMPY	Diazinon
DBCA	Deltamethrin	ALA	Alachlor	DEET	DEET

The quantification of urinary metabolites was done according to the method developed by Olsson et al., 2003(b) at the Pesticide Laboratory, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. The method was as follow: first, 25 µL of labeled internal standard was pipetted into a screw-cap tube; second, 2 mL of urine was pipetted from the urine storage bottle and added to the tube containing internal standard. Then this mixture was well-mixed, using a vortex mixer (Barnstead, IA, USA.). After mixing, 1.5 mL of buffer enzyme solution was added into each tube and inverted gently. The screw caps were replaced and the tubes were placed into an incubator, set at 37˚C for 17 hours. The samples were then cleaned up by solid phase extraction (SPE) in order to maximize the recovery of the target analytes while still removing most of the urine matrix components. The 3 cc Oasis HLB extraction cartridge (Waters, MA.,USA.) was selected for the SPE process. Olsson et al., 2003(b) have previously reported high extraction recoveries (typically >80%) for the target analytes using this SPE cartridge. After the cleanup procedure, the samples analysis was performed on high performance liquid chromatography (HPLC, Agilent 1100, Agilent Tech., Waldbronn, Germany) coupled with the tandem mass spectrometry, using a triple quadrupole mass spectrometer (TSQ 7000, ThermoFinnigan, San Jose, CA, USA) with an atmospheric pressure ionization (API) interface for analyzing the organophosphorus pesticides and selected herbicides, and a triple quadrupole mass spectrometer (Sciex API4000, Applied Biosystems/MDS Sciex, Foster City, CA) for detecting the pyrethroid metabolites.

Quantification and quality control

A calibration curve for quantification was conducted for every analytical run, using 8 different calibration concentrations in blank urine, ranging from 0.25 ng/mL to 50 ng/mL urine.

The 8 calibration curve samples, two fortified urine samples (QC Low and QC High), one blank urine sample, one retention time standard sample and one solvent blank, were prepared, extracted and analyzed in parallel to the unknown sample for each analytical run.

Method validation

Limits of Detection, the LODs, were calculated according to the Quality Assurance of Chemical Measurements written by Taylor (1987). The LODs were equal to three times the

standard deviation of the background noise at zero concentration. This was calculated using the four lowest calibration standards from 7 analytical runs (Taylor, 1987).

Method evaluation

The precision of the method was determined by the analysis over several days of urine pools fortified with analytes at low and high concentration (QC Low and QC High). Relative standard deviations, expressed as a percentage value, were calculated as the standard deviation of the QC pools divided by the mean concentration.

Creatinine measurement

The urine creatinine measurement was performed according to the Roche "Creatinine Plus Assay", using a Roche Hitachi Automatic Analyzer Model 912 (Hitachi, Inc., Japan). The "Creatinine Plus Assay" relies on the chemistry of creatinase and sarcosine oxidase. Through a series of chain reactions in which creatinine is converted to creatine which is in turn converted to sarcosine and urea, sarcosine is oxidized and the hydrogen peroxide is formed. The peroxidase-catalyzed oxidation of a leuko dye by hydrogen peroxide produces a red color whose intensity is measured through absorbance readings and is directly proportional to the creatinine concentration in the sample.

Data analysis

The integration of analytical data was performed both manually and automatically, using XCALIBUR software. Nonparametric analysis of the data as well as the comparisons among groups of farmer were performed, using SPSS version 10.

RESULTS AND DISCUSSION

Method validation

This method has shown a good detection limit (LOD), which ranged from 0.1-0.5 ppb for all specific analytes except for IMPY and CIT. However, the LOD for particular metabolites were 0.1, 0.3, 0.4, 0.2 and 0.1 for PNP, TCPY, t-DCCA, c-DCCA and 3-PBA respectively. According to the limits of detection, especially for organophosphorus pesticide metabolites, this method has LOD values lower than other pervious methods where most of the LODs ranged from 0.1- 1.0 ng/ml [Olsson et al., 2003(a)]. This limit of detection value below 1 ng/mL is generally accepted for the quantification of environmental exposures [Aprea et al., 2002; Barr et al., 2002(b)]. Thus, this method was able to quantify the amount of urinary metabolites from both environmental and occupational exposures. The analytical precision estimated as both within day and between day variations, ranged between 3-14% and 4-19% respectively for the different analytes. This means that this method has shown good precision. The extraction recoveries of analytes ranged between 68-114%. The main advantage of this method over the previous method [Hill et al., 1995; Barr et al., 2002(a); Olsson et al., 2003(a)] was the reduction of sample preparation and cleanup time and the addition of the extra analytes.

Population demographic data

Total of 40 farmers comprised 33 male and 7 female farmers. Their age were ranged

between 19-55 years. These farmers were able to classified depending on their crop types as (1) cut-flower plantation which mostly rose and gerbera were planted (2) vegetable plantation which mostly lettuce, cabbage, kale were planted. Lastly (3) the mixed cultivation of vegetable and cut-flower was planted the same field.

Urinary specific pesticide metabolite monitoring

Because the metabolites of pesticides are usually excreted in urine soon after exposure, biological monitoring of exposure to contemporary pesticides has typically involved qualifying pesticide metabolite in urine [Barr et al., 2002 (b)].

This method was able to quantify 19 selected pesticide metabolites. However, among all urine samples, only 5 metabolites were detected. The descriptive statistical analysis of detected metabolites is demonstrated in Table 2.

The most prevalent compound was PNP which could be detected in all samples. This indicated the methyl parathion and parathion have been used by all farmers. However, it should be noted that concentration of PNP has the highest variation (0.7-2,110.6 μg/g creatinine) which may reflect the different rate of exposure among farmers. Besides, geometric mean and mean concentration of PNP show the highest values compared to the rest of the metabolites. In addition, data in Table 3 indicate that PNP concentration also showed the highest values at all differently-selected percentiles, comparing to TCPY, t-DCCA, c-DCCA and 3-PBA. This indicated that farmers are highly exposed to methyl parathion and parathion. Yet, concentration at selected percentiles of TCPY, t-DCCA, c-DCCA and 3-PBA indicated the same trend and degree of exposure of farmers to chlopyrifos, permetrin, cypermethrin and other pyrethroids which may have resulted from similar exposure.

3-PBA, TCPY, c-DCCA and t-DCCA were detected in 95%, 80%, 80% and 77.5%, respectively, of the total samples. The concentration at selected percentile implied only small amount of exposure. Although significant differences were observed between c-DCCA and t-DCCA *(p=0.00)*, 3-PBA and t-DCCA *(p=0.016)*, 3-PBA and c-DCCA *(p=0.00)*, *compared mean; Wilcoxon*, the mean concentrations still confirmed the low exposure of farmers to chlopyrifos and pyrethroids.

Table 2. Descriptive statistical analysis of detected metabolites in Hmong farmers.

Analytes	Parent Compound	%Detection	Geo.mean (μg./g. cre.)	Mean ±SD (μg./g. cre.)	Min-Max Conc. (μg./g. cre.)
PNP	Parathion	40/40=100	11.8	79.9±333.7*	0.7-2,110.6
TCPY	Chlorpyrifos	32/40=80	2.4	3.4±7.3	<0.3-44.9
t-DCCA	Cyfluthrin,	31/40=77.5	2.5	3.9±6.0**	<0.4-25.4
c-DCCA	Permethrin,	32/40=80	1.1	4.4±14.4**	<0.2-90.2
	Cypermethrin				
3-PBA	18 Pyrethroids	38/40=95	1.5	3.3±5.0***	<0.1-19.9

* The significant differences were observed among PNP and TCPY, t-DCCA, c-DCCA and 3-PBA, *compare mean;Wilcoxon at p=0.00*

** The significant difference was observed between c-DCCA and t-DCCA, *compare mean; Wilcoxon at p=0.00*

*** The significant differences were observed among 3-PBA and t-DCCA, c-DCCA, *compare mean; Wilcoxon at p=0.016 and p=0.000*

Table 3. The concentrations at selected percentile values of detected metabolites.

Analytes	Concentration at selected percentile values (microgram/gram creatinine)					
	10	**25**	**50**	**75**	**90**	**95**
PNP	1.8	5.3	7.0	28.6	104.5	289.4
TCPY	0.0	0.6	1.6	3.7	6.0	13.6
t-DCCA	0.0	0.5	1.7	4.5	11.6	22.9
c-DCCA	0.1	0.2	0.7	3.0	10.0	16.9
3-PBA	0.1	0.6	1.4	3.1	11.6	19.6

Among these detected metabolites, the correlation was observed only between t-DCCA and 3-PBA *(Pearson p=0.00)*, suggesting a common source for these two analytes, likely permethrin or cypermethrin insecticides. The linear regression plot is demonstrated in Graph 1 *(ANOVA, linear regression, p=0.000, r^2=0.953).*

Graph 1. Linear Regression Plot Between t-DCCA vs 3-PBA

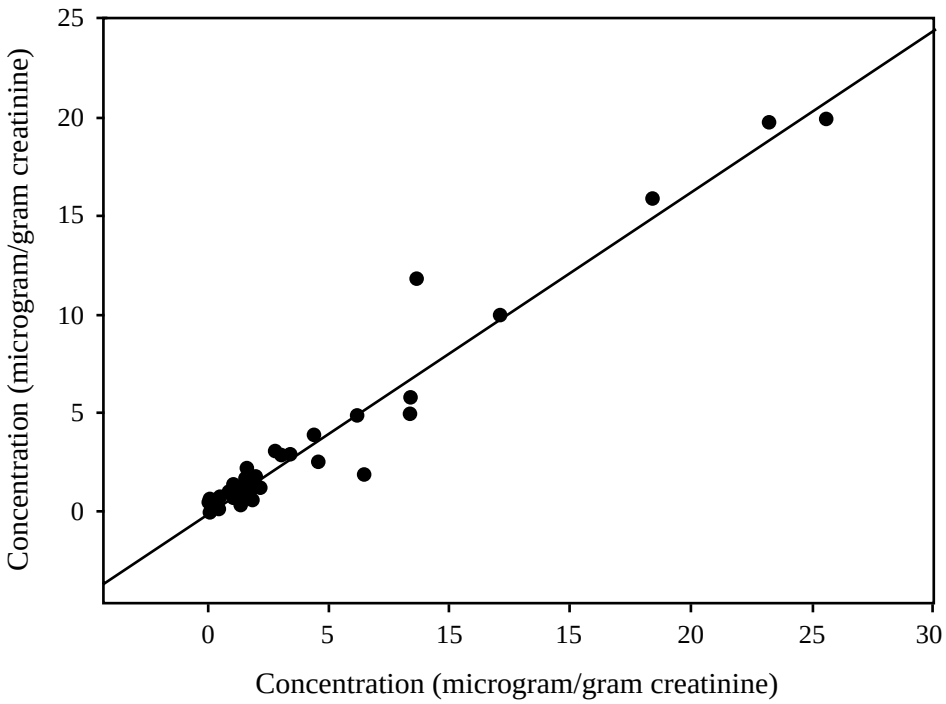

Table 4. Mean concentration of all detected metabolites among farmers in different crop types.

Group	N	Mean Concentration of Metabolites (SD)				
		PNP	TCPY	t-DCCA	c-DCCA	3-PBA
Male	33	86.9 (364.9)	3.5 (7.8)	4.6 (6.4)*	5.3 (15.7)**	3.8 (5.5)
Female	7	46.9 (110.1)	3.0 (4.8)	0.8 (1.1)*	0.4 (0.5)**	1.0 (1.1)
Cut-flower	20	26.1 (64.9)	2.2 (3.16)	5.8 (7.9)	3.3 (4.9)	4.7 (6.5)
Vegetable	11	212.9 (630.1)	6.7 (13.1)	2.2 (2.5)	1.2 (1.3)	2.4 (3.3)
Cut-flower and Vegetable	9	36.8 (53.5)	2.1 (1.2)	2.1 (2.2)	10.8 (29.8)	1.4 (1.1)
Pesticide-free crop	8	1.9 (1.3)***	0.8 (1.4)	1.2 (1.3)	0.5 (0.6)	1.1 (1.6)

* Significant difference, *Mann-Whitney Test, p=0.030*

** Significant difference, *Mann-Whitney Test, p=0.010*

*** Significant differences were found between pesticide-free crop group and all those agricultural types listed in the table *(Mann-Whitney test, p=0.001).*

The mean concentrations of detected metabolites between male and female farmers are shown in Table 4. For PNP, TCPY and 3-PBA in particular, there were no significant differences observed among male and female farmers *(Mann-Whitney Test, p>0.05),* suggesting that those male and female farmers were equally exposed to methyl parathion, parathion, chlorpyrifos and pyrethoids. However, male farmers showed higher exposure to cyfluthrin, permethrin and cypermethrin which resulted in higher mean concentrations of t-DCCA and c-DCCA than female farmers *(Mann-Whitney Test, p=0.030 and p=0.010).*

According to data obtained by questionnaire and household survey documents, farmers could be classified into 3 subgroups, depending on their crop types, namely, farmers who cultivated cut-flower, farmers who cultivated vegetables and farmers who cultivated both cut-flower and vegetables. Mean concentrations of all these three groups were calculated and compared with farmers who cultivated pesticide-free crops used as comparison group also demonstrated in Table 4.

Even though PNP showed its mean concentration quite different for Cut- flower group (26.1 ± 64.9 μg/g creatinine), Vegetable group (212.9 ± 630.1μg/g creatinine), Cut-flower and Vegetable group (36.8 ± 53.5 μg/g creatinine) and Pesticide-free crop group (1.9 ± 1.3 μg/g creatinine), the statistical analysis showed the significant difference only between Pesticides-free crop and Cut-flower group, Vegetable group, Cut-flower and Vegetable group *(Mann-Whitney Test, p=0.001).* No significant difference of PNP was found across Cut-flower group, Vegetable group and Cut-flower and Vegetable group *(Mann-Whitney test, p>0.05).* These results indicate that methyl parathion and parathion are common pesticides used among all groups of farmers except for pesticide-free crop group. However, from the concentrations of detectable PNP, we can infer that those farmers who cultivated pesticide-free crops have been exposed to methyl parathion and parathion by other routes of exposure, such as dietary intake, dermal contact of pesticide in the environment and so forth.

TCPY, t-DCCA, c-DCCA and 3-PBA showed no significant differences in their concentrations across all groups of farmer *(Kruskal Wallis test, $p>0.05$).* Since farmers who were documented as applying pesticides and farmers who were documented as not applying pesticides were equally exposed to cypermethrin, permethrin, cyfluthrin and other pyrethroids, we may conclude that dietary intake and dermal contact of pesticides in environment may be acted as contributing factors. Although statistical analysis shows no significant differences, the mean concentration and SD can indicate less exposure of these certain pesticides among pesticides-free crop group of farmers.

CONCLUSION

Even though this study clearly demonstrates outstanding evidence of pesticide exposure among Thai farmers in a highland agricultural area where pesticides are used on cash crop plantations, further studies are required to establish the reference ranges which in turn may tell us how to determine any pesticide-related health outcomes associated with these exposures.

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