Bull Environ Contam Toxicol (2008) 81:401–405
DOI 10.1007/s00128-008-9515-5

Analytical Method Developed for Measurement of Dialkylphosphate Metabolites in Urine Collected from Children Non-Occupationally Exposed to Organophosphate Pesticides in an Agricultural Community in Thailand

Chidhathai Petchuay · Somkiet Thoumsang · Parichart Visuthismajarn · Banjong Vitayavirasak · Brian Buckley · Paromita Hore · Marija Borjan · Mark Robson

Received: 26 October 2007 / Accepted: 20 August 2008 / Published online: 3 September 2008

Abstract There has been increasing concern in regards to organophosphate (OP) pesticide exposure among farm workers and their families in Thailand's agricultural areas. Therefore, the development of an analytical method for estimating OP pesticide exposure is necessary to allow for monitoring of OP pesticide exposures within these populations. This paper describes an analytical method developed to measure dialkylphosphate (DAP) metabolites in urine. The methods in this study are important in the biological monitoring of OP metabolites in agricultural families in Thailand and can be used as an initial guidance procedure in any environmental toxicological laboratory in Thailand.

Keywords Dialkyphosphate · Analytical method · Non-occupational exposure · Organophosphate · Pesticide

C. Petchuay
Department of Biological Science, Faculty of Science, Ubon Ratchatani University, Ubon Ratchatani 34190, Thailand

C. Petchuay
National Research Center for Environmental and Hazardous Waste Management (NRC-EHWM), Chulalongkorn University, Bangkok 10330, Thailand

S. Thoumsang
Reference Laboratory and Toxicology Center, Bureau of Occupational and Environmental Disease, Department of Disease Control, Ministry of Public Health, Nontaburi 11000, Thailand

P. Visuthismajarn · B. Vitayavirasak
Faculty of Environmental Management, Prince of Songkla University, SongkhlaHat Yai, 90112, Thailand

B. Buckley
Environmental and Occupational Health Sciences Institute, Piscataway, NJ 08854, USA

P. Hore · M. Borjan · M. Robson
School of Public Health, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, USA

M. Borjan · M. Robson (✉)
The New Jersey Agricultural Experimental Station, Rutgers University, New Brunswick, NJ 08901, USA
e-mail: robson@aesop.rutgers.edu

A large amount of pesticide active ingredients are routinely imported into Thailand for controlling pests and increasing crop production in the agricultural sector (Integrated Pest Management Thailand 2003). Organophosphate (OP) insecticides are widely applied by Thai farmers. Consequently, there has been an increasing level of concern with OP pesticide exposures for farm workers and their families living in agricultural areas. Therefore, development of an analytical method for estimating OP pesticide exposure is necessary to monitor OP pesticide exposures within these population groups.

In Thailand, the main method of monitoring human exposure to OP pesticides has typically involved measurement of blood cholinesterase enzyme activity. This method, however, is less sensitive for low level OP exposures that can occur in occupational and non-occupational settings. One approach that has been widely accepted in evaluating OP exposures is the measurement of biomarkers of exposure (Barr and Needham 2002). Various studies have been reported which use measurements of urinary dialkylphosphate (DAP) metabolites of OP pesticides to estimate human exposure to OP pesticides (Aprea et al. 1996; Moate

et al. 1999; Fenske et al. 2000; Hardt and Angerer 2000; Lu et al. 2001; Oglobine et al. 2001a, b; Bravo et al. 2004). Six common DAP metabolites consist of dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP) and diethyldithiophosphate (DEDTP). The chemical structures of the six DAP metabolites are shown in Fig. 1.

Several published methods have been developed for quantifying DAP metabolites. One of the most important steps in analyzing urinary DAP metabolites is metabolite isolation from the urine matrix. The extraction techniques reported in the literature include using liquid–liquid extraction with polar solvents (Hardt and Angerer 2000), azeotropic distillation (Moate et al. 1999), and lyophilization (Oglobine et al. 2001a, b; Bravo et al. 2004). Amongst the reported techniques, lyophilization is the most robust and convenient method that can be used to isolate DAP metabolites from urine. This technique gives high DAP recovery and reduces gummy residue during sample preparation (Bravo et al. 2004). Following lyophilization, a derivatization procedure is required. DAP metabolites can be derivatized to make them more volatile using various reagents, including diazomethane, diazopentane which cannot be used for an accurate analysis of dimethylphosphate (Barr and Needham 2002), 1-chloro-3-iodo-propene (Bravo et al. 2004), and pentafluorobenzyl bromide (PFBBr). PFBBr is the most commonly used derivatizing agent because the literature has shown it gives good results (Nutley and Cocker 1993; Moate et al. 1999; Aprea et al. 1996; Hardt and Angerer 2000; Oglobine et al. 2001a, b). The derivatized extracts can be analyzed using GC coupled with several detection methods, which include flame photometric detection (FPD) (Aprea et al. 1996; Moate et al. 1999; Oglobine et al. 2001b), flame ionization detection (FID), mass spectrometry (GC-MS) (Hardt and Angerer 2000) or tandem mass spectrometry (GC-MS-MS) (Oglobine et al. 2001a; Bravo et al. 2004).

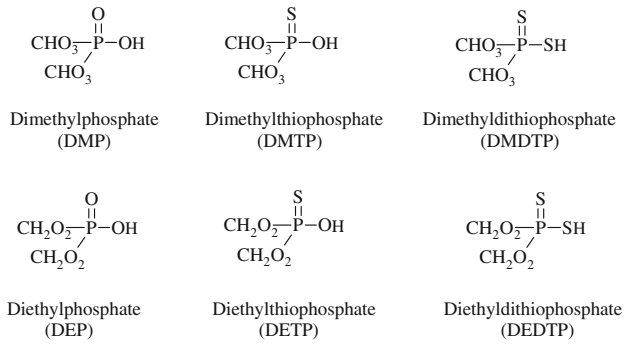

Fig. 1 Chemical structures of the six dialkylphosphate metabolites

Because mass spectrometry and tandem mass spectrometry are not routinely available in Thai laboratories, methods based on more affordable detectors were needed. Our aim was to couple the most effective sample preparation techniques with affordable analytical platforms to allow the routine measurement of DAP metabolites in exposure assessment studies in Thailand.

Materials and Methods

Most of the standard reagents for this study were obtained commercially. DMP, DMTP, DETP, and DEDTP as the potassium salt, all at 98% purity, were obtained from Aldrich Chemical Co., Germany; DEP also with 98% purity was obtained from ACROS Organics, NJ USA. A DMDTP standard was not available commercially. PFBBr at 99% purity was obtained from Aldrich, USA; anhydrous potassium carbonate (K_2CO_3) was obtained from Merck, Germany. Dibutylphosphate (DBP) used as an internal standard was purchased from Fluka Chemie, Switzerland.

The GC used for urine analysis (Hewlett Packard 6890) was equipped with a flame photometric detector (Hewlett Packard 19256A GC-FPD). A capillary column, HP5 (Hewlett Packard part no. 19091J-433) 5% Phenyl Methyl Siloxane (30 m × 0.32 mm id × 0.25 μm film thickness) was used to separate the analytes. Sample injection (1 μL) was performed in splitless mode. The temperature of the injector was set at 250°C. The oven temperature was programmed to start at an initial temperature of 80°C held for 2 min, then ramped to 210°C at a rate 17°C/min (Run time = 12.67 min).

Lyophilization was performed using a Dura-Top freeze-dryer (Vendor information). A 5 mL aliquot of each urine sample was pipetted into individually labeled screw cap glass test tubes and spiked with 100 μL of 10 mg/L internal standard (DBP). The samples were then mixed and frozen at −40°C. After the samples were adequately frozen, they were lyophilized with the Dura Top freeze-dryer for ∼6–7 h.

After the freeze drying process, 2 mL of acetronitrile, 30 mg of anhydrous potassium carbonate and 25 μL of PFBBr were added to each freeze-dried urine tube to derivitize the samples. The samples were then mixed and heated at 60°C for 3 h in a water bath. Subsequently, the supernatant was transferred to a clean tube and evaporated to dryness with a gentle nitrogen stream. The samples were then cooled, transferred to small vials, and stored under refrigeration until analysis.

A standard stock solution was prepared by dissolving appropriate quantities of DMP, DEP, DMTP, DETP, and DEDTP standard, in acetonitrile to reach a final concentration of 10 mg/L. Working standard solutions were

prepared in five concentration levels of mixed DAP, ranging from 10–500 μg/L of urine. The standard curves were prepared by using urine from unexposed persons. Finally, 100 μL of DBP was added to each standard solution. Correlation coefficient (r^2) values for the calibration curves were ≥0.996 indicating good linearity for each metabolite.

Results and Discussion

An optimal method for analyzing DAP urinary metabolite was developed in this study based on the procedures described by Oglobine et al. (2001a, b) and Bravo et al. (2004). The technique of lyophilization was used to remove water from the urine samples. This technique is less labor-intensive and produces better or more consistent recoveries for derivatized analytes compared to other techniques. The freeze drying process in this study took only 6–7 h to achieve complete urine dryness after the samples were frozen. The PFBBr derivatization in acetronitrile was selected to isolate the DAP metabolites from the urine residue because the PFBBr reagent formed only one reaction product with each metabolite, and the derivatizes were soluble in the acetonitrile layer adequately separating them from the remaining residue (Oglobine et al. 2001a).

Reaction temperature and time were also important factors during the derivatization-assisted extraction of DAP metabolites since the alkylation of DAP metabolites is a thermosensitive reaction and high temperature degradation of the sulfur-containing DAP metabolites, DMTP, DMDTP, DETP, and DEDTP can occur (Moate et al. 1999; Oglobine et al. 2001a, b). The method presented here performed the derivatization at 60°C for 3 h as suggested by Bravo et al. (2004). These conditions were optimal and yielded all of the DAP metabolites in one derivatization step without the loss of sulfur-containing compounds.

This study employed a GC-FPD for measuring the DAP metabolites. GC-FPD has been used to analyze DAP metabolites in various studies of OP pesticide exposure for children and adults (Aprea et al. 1996; Moate et al. 1999). Under the experimental conditions reported here, the DAP metabolites had retention times of DMP 7.14 min, DEP 7.96 min, DMTP 8.28 min, DETP 8.9 min, DEDTP 9.35 min and DBP 9.97 min. Chromatograms of a DAP standard spiked urine sample and an actual child's urine sample are presented in Figs. 2 and 3, respectively.

The limit of detection (LOD) was calculated for each of the DAP metabolites. LODs for the DAP metabolites ranged from 1 to 5 μg/L (Table 1). Although, the LODs are higher than those previously reported using lyophilization coupled with GC/MS-MS which range from 0.02 to 0.5 (Oglobine et al. 2001b) and 0.1–0.6 μg/L (Bravo et al. 2004) likely because of the added selectivity and sensitivity of mass spectrometric detection, they are lower than those previously reported by others using the freeze dry process with GC-FPD detection (5–50 μg/L) (Oglobine et al. 2001a). More importantly, however, they are suitable to assess OP pesticide exposure in child studies resulting from agriculturally-related exposures (Loewenherz et al. 1997), even as we have observed in our study of children in a Southern Thailand agricultural community (vide infra).

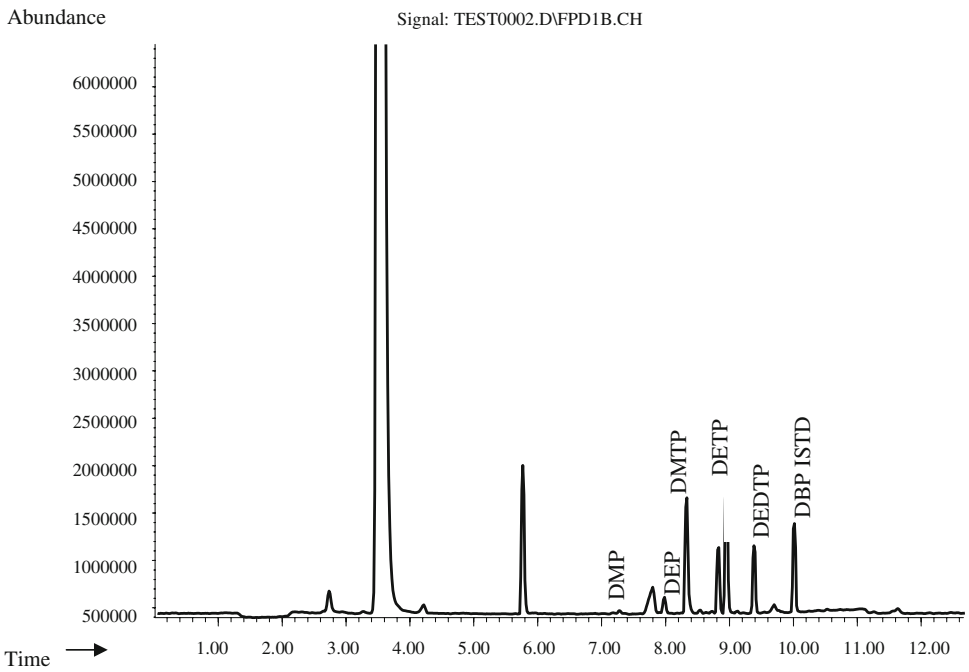

Fig. 2 Chromatogram of a blank urine sample spiked with a standard solution containing five DAP metabolites. *Note*: DAP metabolite DMDTP was not available for standard preparation

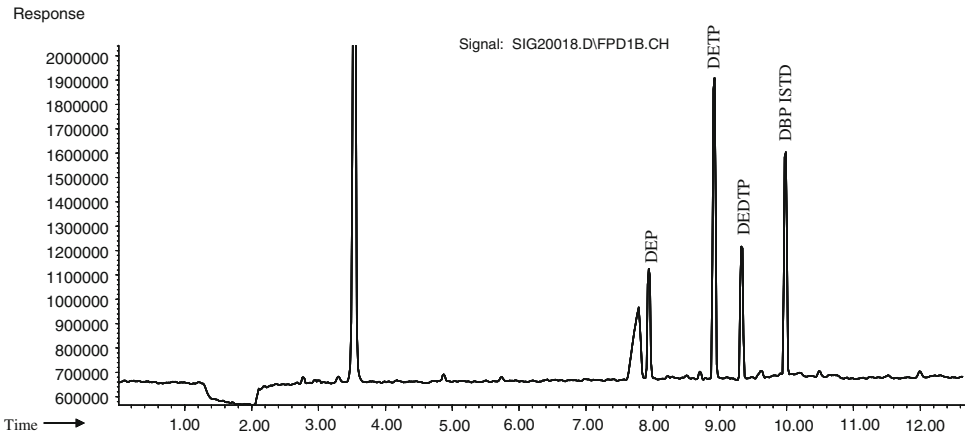

Fig. 3 Chromatogram of a non-occupationally exposed child's urine sample

Table 1 Limits of detection (LOD) and recovery of the DAP metabolites

Analyte	LOD (μg/L)	Recovery (%)	LOD (μg/L)[a]	Recovery (%)[a]
DMP	5	60.0	5	ND[b]
DEP	3	67.5	3	43.0
DMTP	1	107.5	3	113.0
DETP	1	98.6	1	97.0
DEDTP	3	90.3	1	85.0

[a] From Weisskopf and Seiber (1989)

[b] The procedures in the Weisskopf and Seiber paper are unsuitable for DMP due to interferences caused by the presence of inorganic phosphate

Recovery studies were conducted by preparing each DAP standard in blank urine at 10, 50 and 100 μg/L and carried out with the procedure of this study. The method gave the following derivatization-assisted extraction recoveries of 60% DMP, 68% DEP, 107.5% DMTP, 98.6% DETP, and 90.3% DEDTP (Table 1). The derivatization conditions at 60°C for 3 h as suggested by Bravo et al. (2004) were the optimal reaction conditions for this study.

Among other derivatization methods using FPD, the method by Weisskopf and Seiber used a solid phase extraction cartridge to isolate the metabolites. Solid phase methods use a sorbent material to isolate the metabolites and then elute them from the column or filter material. The method by Weisskopf and Seiber demonstrated reasonably good recoveries of a fortified urine sample for all metabolites other than DEP. In addition, DMP could not be measured in actual samples because of its presence in the solvent blank. Using a solid phase method does have advantages in its ability to perform a cleanup step before derivatization, leaving many potential interferents behind but was not able to overcome the persistent DMP in the blanks. A greater discussion of blank problems associated with SPE protocols can be found in Stiles et al. (2007). Weisskopf and Seiber also demonstrated the use of TMAH as a derivatizing agent. The detection limits with this overall method were roughly 10 μg/L for DEP and 2 μg/L for the other metabolites although at 2 μg/L only 48% of the DEDTP was recovered.

The method described in this work uses a lyophilization technique and derivatization with pentafluorobenzyl bromide (PFBBr). The optimal procedures were adapted based on some previously published procedures described by Oglobline et al. (2001a, b) and Bravo et al. (2004). Both this work and the previous method use FPD to measure the eluates, so it is believed any sensitivity differences will lie in either sample preparation or derivatization. The LODs using lyophilization and PBBR derivatization were comparable or better than the method by Weisskopf and Seiber. Specifically, the LODs were 5 μg/L DMP (dimethylphosphate), 3 μg/L DEP (diethylphosphate), 3 μg/L DMTP (dimethylthiophosphate), 1 μg/L DETP (diethylthiophosphate), and 1 μg/L DEDTP (diethyldithiophosphate).

We used this method to assess OP pesticide exposure among children in a Thai agricultural community. In this study, urine samples were collected from 37 children (2–5 year old) living in or near a vegetable farm. The average ages of the participating children were 3.7 years including 3.41 years for female ($n = 17$) and 3.9 years for male ($n = 20$). The urine samples were collected typically during the months of the high spraying season, April–March. At least one of the DAP metabolites was detected in each child urine sample collected with concentrations ranging from <LOD to 22.8 μg/L for volume-adjusted concentrations, or <LOD to 48.6 μg/g creatinine for creatinine-adjusted concentrations. The DAP metabolites DEP and DETP were detected with the greatest frequency in the farm children's urine samples (57% and 38%, respectively). The study also found that children living inside or near farm areas had significantly higher

levels of DAP metabolites, such as DMP, DMTP, DETP and DEDTP, compared to the reference children living outside farm areas.

We describe a usable, robust method for measuring urinary DAP metabolites as indicators of exposure to OP pesticides. This method uses a simple preparation method and improved derivatization chemistry to create a relatively sensitive measurement technique for OP metabolites by GC with non mass spectrometric detection. More importantly, the ability to obtain a basic and simple yet sensitive measurement of OP pesticide exposure is important because among populations where exposure to OP pesticides is more likely to occur, there is often a lack of more sophisticated instrumentation. This method using GC with FPD with the sensitivity described here allows for the measurement of these metabolites among the population that needs it most. We have demonstrated the utility of the method by measuring DAP concentrations in a Thai child population living in an agricultural community.

Acknowledgements This study was supported by National Research Center for Environmental and Hazardous Waste Management (NRC-EHWM), Chulalongkorn University. The authors also thank the Reference Laboratory and Toxicology Center, Bureau of Occupational and Environmental Disease, Department of Disease Control, Ministry of Public Health, for providing the facilities and assistance in developing method for dialkylphosphate metabolite measurement. We also thank Dr. Dana Barr for her helpful suggestions in revising this manuscript. Support was also provided by NIEHS P30 ES05022.

References

Aprea C, Sciarra G, Lunghini L (1996) Analytical method for the determination of urinary alkylphosphates in subjects occupationally exposed to organophosphorus pesticides and in the general population. J Anal Toxicol 20:559–563

Barr DB, Needham LL (2002) Analytical methods for biological monitoring of exposure to pesticides: a review. J Chromatogr B 778:5–29. doi:10.1016/S1570-0232(02)00035-1

Bravo R, Caltabiano LM, Weerasekera G, Whitehead RD, Fernandez C, Needham LL, Bradman A, Barr DB (2004) Measurement of dialkyl phosphate metabolites of organophosphorus pesticides in human urine using lyophilization with gas chromatography-tandem mass spectrometry and isotope dilution quantification. J Expo Anal Environ Epidemiol 14:249–259. doi:10.1038/sj.jea.7500322

Fenske RA, Lu C, Simcox NJ, Loewenherz JT, Moate TF, Allen EH, Kissel JC (2000) Strategies for assessing children's organophosphorus pesticide exposures in agricultural communities. J Expo Anal Environ Epidemiol 10:662–671

Hardt J, Angerer J (2000) Determination of dialkyl phosphates in human urine using gas chromatography-mass spectrometry. J Anal Toxicol 24:678–684

Integrated Pest Management (IPM) Thailand (2003) Did you take your poison today? A report by the IPM DANIDA project. http://www.ipmthailand.org

Loewenherz C, Fenske RA, Simcox NJ, Bellamy G, Kalman D (1997) Biological monitoring of organophosphorus pesticide exposure among children of agricultural workers in central Washington State. Environ Health Perspect 105:1344–1353

Lu C, Knutson DE, Anderson JF, Fenske RA (2001) Biological monitoring of organophosphorus pesticide exposure among preschool children in the seattle metropolitan area. Environ Health Perspect 109:299–303. doi:10.2307/3434700

Moate TF, Lu C, Fenske RA, Hahne RMA, Kalman DA (1999) Improved cleanup and determination of dialkyl phosphates in the urine of children exposed to organophosphorus insecticides. J Anal Toxicol 23:230–236

Nutley BP, Cocker J (1993) Biological monitoring of workers occupationally exposed to organophosphorus pesticides. Pestic Sci 38:315–322. doi:10.1002/ps.2780380406

Oglobine AN, Elimelakh H, Tattam B, Geyer R, O'Donnell GE, Holder G (2001a) Negative ion chemical ionization GC/MS-MS analysis of dialkylphosphate metabolites of organophosphate pesticides in urine of non-occupationally exposed subjects. Analyst 126:1037–1041. doi:10.1039/b102004h

Oglobine AN, O'Donnell GE, Geyer R, Holder GM, Tattam B (2001b) Routine gas chromatographic determination of dialkylphosphate metabolites in the urine of workers occupationally exposed to organophosphorus insecticides. J Anal Toxicol 25:153–157

Stiles R, Yang I, Lippincott RL, Murphy E, Buckley B (2007) Identifying potential sources of background contaminants resulting from solid phase extraction and solid phase microextraction. J Sep Sci 30:1029–1036. doi:10.1002/jssc.200600358

Weisskopf CP, Seiber J (1989) New approaches to analysis of organophosphate metabolites in the urine of field workers. In: Wang RG, Franklin CA, Honeycutt RC, Reinert C (eds) Biological monitoring for pesticide exposure. Measurement, estimation and risk reduction. American Chemical Society, Washington DC, pp 206–214