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Short communication

Revised method for routine determination of urinary dialkyl phosphates using gas chromatography–mass spectrometry☆

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ABSTRACT

Among urinary organophosphorus pesticide (OP) metabolites, dialkyl phosphates (DAPs) have been most often measured as a sensitive biomarker in non-occupational and occupational OP exposure risk assessment. In our conventional method, we have employed a procedure including simple liquid–liquid extraction (diethyl ether/acetonitrile), derivatization (pentafluorobenzylbromide, PFBBr) and clean-up (multi-layer column) for gas chromatography–mass spectrometry (GC–MS) analysis starting from 5-mL urine samples. In this study, we introduce a revised analytical method for urinary DAPs; its main modification was aimed at improving the pre-derivatization dehydration procedure. The limits of detection were approximately 0.15 μg/L for dimethylphosphate (DMP), 0.07 μg/L for diethylphosphate (DEP), and 0.05 μg/L for both dimethylthiophosphate (DMTP) and diethylthiophosphate (DETP) in 2.5-mL human urine samples. Within-run precision (percent of relative standard deviation, %RSD) at the DAP levels varying in the range of 0.5–50 μg/L was 6.0–19.1% for DMP, 3.6–18.3% for DEP, 8.0–25.6% for DMTP and 9.6–27.8% for DETP. Between-run precision at 5 μg/L was below 15.7% for all DAPs. The revised method proved to be feasible to routine biological monitoring not only for occupational OP exposure but also for environmental background levels in the general population. Compared to our previous method, the revised method underscores the importance of adding pre-derivatization anhydration for higher sensitivity and precision.

1. Introduction

Organophosphorus compounds (OPs) have been widely and effectively used as insecticides with applications in agricultural settings, public health, commerce, and individual households throughout the world [1]. Growing concern over the long-term effects of low-level exposure to OPs on human health has encouraged more detailed research both in experimental and in epidemiological settings. Since about 75% of the US Environmental Protection Agency (EPA)-registered OPs are metabolized to dialkyl phosphates (DAPs), including dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP), and diethyldithiophosphate (DEDTP) [2,3], these DAPs in urine have been measured as biomarkers of OP exposure [4,5]. Development of analytical equipment and protocols of sample preparation has made it possible to detect low-level DAPs from various general populations, revealing that OP exposure has commonly occurred even in ordinary daily life. However, the main pathway of human exposure (e.g. ingestion, inhalation or dermal absorption) as well as toxicity due to long-term exposure to low-level doses still remains to be explored. This is partly because of the difficulty in determining low-level DAPs among biological samples obtained from the general population, due to the complicated and costly sample preparations, overall low-throughput yield, and requirement of expensive equipment. Urine sample has been frequently used for

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the determination of DAP because they are collected non-invasively and contain DAPs in higher concentrations than other biological samples [6,7].

We previously reported a method for DAP analysis [8], which is relatively simple and sensitive enough to be adopted in routine biological monitoring of non-occupational as well as occupational exposure to OPs. However, there were still some shortcomings to be resolved such as low precision and the need for a relatively large volume of urine (>5 mL). The aim of the present study was to improve our previous methods [8] for urinary DAP measurement using gas chromatography–mass spectrometry (GC–MS) equipped with an electron ionization system and achieve higher sensitivity and precision with high throughput and lower cost.

2. Experimental

2.1. Reagents

DMP tetramethylammonium salt (99.9% purity), DMTP ammonium salt (98.9%), DEP (98.2%) and DETP ammonium salt (95.2%) were obtained from Hayashi Pure Chemical Ind. (Osaka, Japan), and dibutylphosphate (DBP), used for an internal standard (I.S.), was from Tokyo Kasei Kogyo (Tokyo, Japan). Diethyl ether, acetonitrile, *n*-hexane, acetone and toluene, which are pesticide residue grade, and sodium sulfate anhydrous, sodium chloride (NaCl), sodium disulfite ($Na_2S_2O_5$) and 6 M hydrochloric acid, were purchased from Kanto Chemicals (Tokyo, Japan). Pentafluorobenzylbromide (PFBBr) was purchased from Sigma–Aldrich (St. Louis, MO, USA), Florisil (60–100 mesh) from Wako Pure Chemicals (Osaka, Japan), and Primary/Secondary Amine (PSA) 40 μm from Varian, Inc. (Palo Alto, CA, USA). Water used throughout the experiments was distilled and deionized to 18 MΩ with a Millipore Milli-Q System (Millipore Co., Bedford, MA, USA). All other reagents were of analytical grade purity. Muromac Mini Column (medium size, 110 mm × 8.0 mm i.d.) (Muromachi Chemical Inc., Fukuoka, Japan) was used for dehydration and clean-up process. The clean-up column could be readily prepared without any special technique and was composed of 0.3 g of Florisil (lower), 0.1 g of PSA (middle) and 0.5 g of sodium sulfate anhydrous (upper).

2.2. Apparatus and GC–MS conditions

Analyses of DAPs derivatized with pentafluorobenzylbromide were performed using an Agilent 5975 inert MSD system. The GC operating conditions were as follows: GC column, Rtx-65 (Restek, USA), 30 m × 0.25 mm i.d., 0.25-μm film thickness; column temperatures, 70 °C (1 min)–15 °C/min–300 °C (6 min); injection port temperature, 250 °C; carrier gas, helium (99.999% purity); flow rate, 1 mL/min. The injection volume was 1 μL. Splitless was changed to split 15:1 at 2 min after sample injection. The MS operating conditions were as follows: ionization source temperature, 230 °C; electron ionization, 70 eV; interface temperature, 300 °C; injection pressure, 88 psi. Chromatogram peak was identified by target and quantifier ions for each pentafluorobenzyl (PFB)-DAP as shown in Table 1. Use of C- and Q-ion presented in this table is appropriate for selectivity and sensitivity under new analytical conditions.

2.3. Standard preparation and analytical procedure

Each standard (DMP, DEP, DMTP and DETP) was prepared at a concentration of 1000 mg/L in methanol, and diluted with methanol to each working standard solution. The standard solutions were stored at 4 °C in the dark, and were used within 1 month of their preparation. Urine samples from healthy volunteers, who were neither treated with any drugs nor exposed to chemicals before collection, were used for the basic methodological examination in this study.

A flow chart of the urinary DAPs determination procedure is shown in Fig. 1. Urine sample (2.5 mL) was pipetted into a 10-mL screw-top glass test tube, and 20 μL of I.S. solution (100 mg/L DBP), 2.5 g of NaCl, 1 mL of 6 M HCl, 50 mg of $Na_2S_2O_5$ and 2.5 mL of diethylether–acetonitrile (1:1, v/v) were added. After vigorous mechanical shaking for 5 min, the test tube was centrifuged (1500 × *g* for 5 min at room temperature). The organic phase (upper layer) containing DAPs was passed through 1 g of anhydrous sodium sulfate column and collected into a new screw-top glass test tube containing 15 mg of K_2CO_3. The aqueous phase was re-extracted with 2.5 mL of diethylether–acetonitrile (1:1, v/v) and then centrifuged. The supernatant obtained from the second extraction was passed through the anhydrous sodium sulfate column, and combined with the first extract. The resulting extract was evaporated at 40 °C (heat block) to dryness with a gentle nitrogen stream for about 1 h. To the dried extracts, 15 mg of K_2CO_3, 1 mL of acetonitrile and 10 μL of PFBBr were added and incubated in a heat block at 80 °C for 30 min with occasional swirling. Afterwards, 3 mL of water and 3 mL of *n*-hexane were added, and the mixture was shaken vigorously for 5 min and centrifuged for 5 min (1500 × *g* for 5 min). The upper layer containing PFB-DAPs was transferred to new test-glass tubes. The extraction was then repeated with 3 mL of *n*-hexane, and the supernatant obtained from the second extraction was combined with the first extract. The combined extract was loaded into a clean-up column, followed by washing with 5 mL of acetone-*n*-hexane (2:98, v/v) for removing unreacted PFBBr. PFB-DAPs were then eluted with 5 mL of acetone-*n*-hexane (15:85, v/v), and the eluate was evaporated at 45 °C to dryness with a gentle nitrogen stream for about 15 min. The residue was dissolved in 200 μL of toluene and injected into GC–MS.

2.4. Assay validation

Using the proposed method, two calibration curves were separately prepared using pooled urine. The first curve corresponded to the concentrations of urinary DAPs ranging from 0.5 to 50 μg/L (four points), and the second curve to the range from 50 to 1000 μg/L (five points); the latter curve was used along with the former one to determine the urinary DEP, DMTP and DETP in occupational OP exposures.

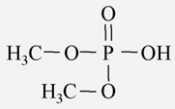
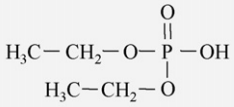
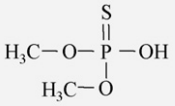
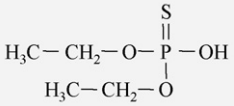
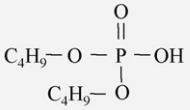
To determine and calculate absolute recoveries, we spiked DAPs at two different stages in the procedure; i.e. in the beginning of the extraction procedure (urine sample) and prior to the derivatization procedure. We compared the I.S. ratios obtained at these two stages.

Calibration curves were represented by the analyte/I.S. peaks area ratio versus the concentrations of the calibration samples. The within-run precision for our revised method was examined through the assay of the pooled urine spiked with DAP concentrations of 0.5, 2.5, 5 and 50 μg/L (n = 4–5). Moreover, the between-run precision was examined through the duplicate assay of the pooled urine spiked with DAPs at a concentration of 1, 5 and 500 μg/L for 4 consecutive days (n = 3–4). The limits of detection (LOD) and limit of quantitation (LOQ) were calculated based on the signal-to-noise ratio of 3 and 10, respectively.

2.5. Application of methods to field study samples

DAPs were measured in morning urine samples collected from 25 healthy OP non-exposed volunteers aged 40 ± 9 years (mean ± S.D.) in February 2009 (low-exposure urine). The methods were also applied to morning urine samples collected from 25 OP exposed persons aged 37 ± 9 years (mean ± S.D.) in August 2009 (high-exposure urine); they were all workers engaged in pest control occupation (PCO) located in the Chubu area (central Japan) and

Table 1
Chemical structures, fragment ions and retention time of dialkyl phosphates and dibutyl phosphate.

Compound	Structure	m/z[a]		Retention time (min)
		C-ion[b]	Q-ion[c]	
DMP		110	306	8.86
		194		
DEP		258	334	9.44
		197		
DMTP		322	322	10.28
		211		
DETP		213	350	10.73
		274		
DBP (internal standard)		335	335	11.52

[a] m/z: mass/charge ratio.
[b] C-ion: selected ions for confirmation.
[c] Q-ion: selected ions for quantification.

asked to provide urine samples collected on the day after insecticide spraying. The reason for the difference in the sample collection season was to verify whether the present method is feasible for a wide range of DAP concentrations; pesticide exposure is lowest in the general population in winter [9] and highest in PCOs in summer [10]. Collected urine samples were immediately transferred into 10 mL polyethylene tubes and stored at −80 °C until DAP assay. The Ethics Committee of the Nagoya University Graduate School of Medicine and Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan approved the study protocol. When urinary DAP concentrations were less than LOD, they were estimated as half the LOD value for statistical analyses [11].

3. Results and discussion

3.1. Assay validation

The validation parameters are summarized in Table 2. For the within-run precision, percent of relative standard deviation (%RSD) ranged from 5.6 to 27.8% for all DAPs. For the between-run precision, the %RSD was between 7.0 and 51.3%. Reproducibility deteriorated when the analyte/I.S. peak area ratio was not used (data not shown). Relatively high %RSD values were shown for the within-run precision at 0.5 μg/L (DMTP 25.6% and DETP 27.8%) and between-run precision at 1 μg/L (DMP 36.7%, DMTP 51.3% and DETP 23.0%). The urinary DETP concentration of 0.5 μg/L is near the geometric mean in the general population [5,12]. On the other hand, geometric means of urinary DMP and DMTP level in the general population are around 10 μg/L or more [12,13]. Therefore, biomonitoring of DETP at low concentrations is less precise in the assessment of the OP exposure level for the general population. Further studies are needed to accurately determine the urinary DETP concentrations in the general population. Absolute recovery data are approximately similar to those of our previous report [8], and superior to the solid-phase extraction recovery except for DEP [14]. The mean absolute recoveries for DMP were lower than those for other DAPs due to its high polarity. This lower recovery might increase the LOD value for DMP. Because absolute recoveries of DAPs in water differ from those in urine matrix (data not shown), pooled urine should be used for a matrix matched calibration curve. LOD and LOQ values are below or similar to those reported by De Alwis et al. [14,15], who used solid-phase extraction and GC–MS/MS, Dulaurent et al. [16], who used liquid–liquid extraction and LC–MS/MS, or by Ueyama et al. [8]. Concentrations of LOQ are lower than the geometric mean of urinary DAPs in the general populations including children and pregnant women [12,17–19], suggesting that our present method is sensitive enough for monitoring urinary DAPs in most general populations. The ratios of C- and Q-ion abundances for each DAP were almost invariable at concentrations both above and below LOD. It is unlikely that some interference substances deteriorate selectivity for urinary DAP concentrations below LOD.

3.2. Advantages of this method

Advantages of the present method over the previous one were smaller sample volume required without compromising the high sensitivity and precision, cost-effectiveness and smaller volume of such reagents as diethylether/acetonitrile mixture, NaCl and irritating nature PFBBr.

Previously, urinary DAPs have been extracted by various methods: liquid–liquid extraction [8,20,21], solid-phase extraction [14,15,22,23], and lyophilization [24]. Weerasekera et al. [3] suggested that solid-phase extraction (ChemElute cartridge) and

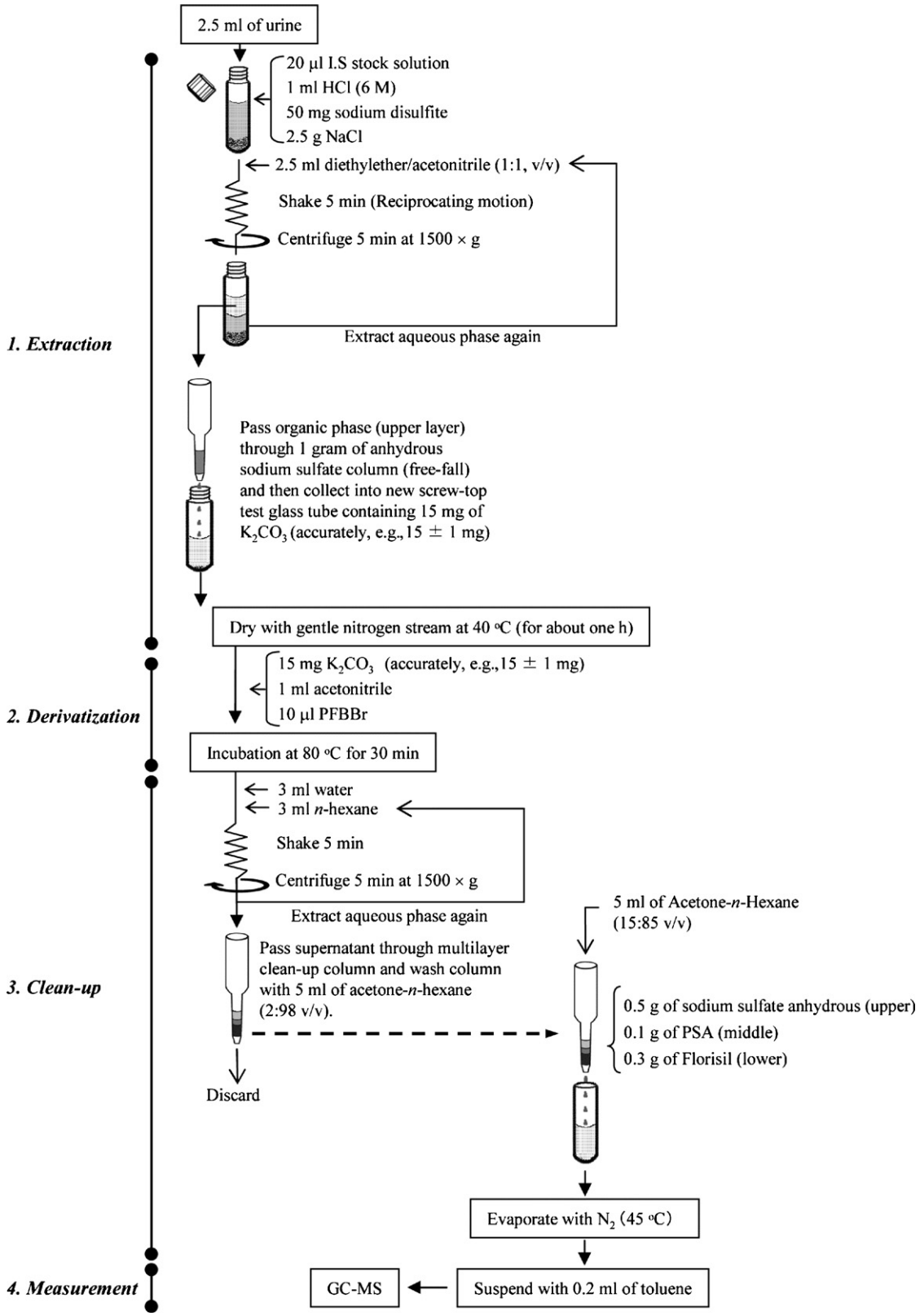


Fig. 1. Analytical procedure for urinary DAPs.

analysis using the GC–MS is the most cost-effective and rapid method. There were some drawbacks to other extraction methods; lyophilization is time-consuming and liquid–liquid extraction is less accurate and precise [3]. If the accuracy and precision are remediated, it should be possible to develop the liquid–liquid extraction as a more cost-effective and highly sensitive DAP extraction procedure. We hypothesized that the derivatization procedure using PFBBr would be adversely affected by any interference substance in extracts after the liquid–liquid extraction procedure. It is well known that the derivatization reaction of PFBBr is

Table 2
Accuracy, precision, LOD and LOQ data of analytical procedure.

	Pooled urine spiked concentration (μg/L of urine)	n[a]	DMP	DEP	DMTP	DETP
Within-run						
Precision (%RSD[b])	0.5	5	19.1	18.3	25.6	27.8
	2.5	4	7.6	6.3	8.0	12.3
	5	4	5.6	4.1	12.5	15.1
	50	4	6.0	3.6	15.6	9.6
Mean recovery[e] (%)	1	4	62.6	87.9	84.0	102.0
	50	4	68.6	85.6	88.4	97.7
	500	4	71.3	90.5	83.5	91.6
Between-run						
Precision (%RSD)	1	3	36.7	16.4	51.3	23.0
	5	4	15.7	10.3	9.3	7.0
	500	3	10.2	15.1	9.9	11.3
R^2 of calibration line						
0.5–50 μg/L of urine			0.995	0.998	0.984	0.974
50–1000 μg/L of urine			0.973	0.980	0.991	0.989
LOD[c] (μg/L) (signal-to-noise ratio = 3)			0.15	0.07	0.05	0.05
LOQ[d] (μg/L) (signal-to-noise ratio = 10)			0.5	0.3	0.2	0.2

[a] *n*: number of observations.
[b] RSD: relative standard deviation.
[c] LOD: limit of detection.
[d] LOQ: limit of quantitation.
[e] Recovery given by adding the standards on derivatization step.

inhibited in the presence of water. In the extraction procedure, the organic phase obtained from liquid–liquid extraction using diethylether–acetonitrile (1:1, v/v) may contain much water. The water retained in high hydroscopic solid K_2CO_3 could have affected the efficiency of derivatizing DAPs with PFBBr, thus resulting in the deteriorated validation data even if the post-derivatization dry up procedure was conducted. Therefore, we examined how the dehydration procedure prior to PFBBr derivatization affects the accuracy, precision and sensitivity. The improvement found in our validation data is mainly attributable to the pre-derivatization dehydration. For example, within-run precision (RSD%) concentrations at 5 μg/L in the present method (5.6 for DMP and 4.1 for DEP) were lower than those in our previous method (16.1 for DMP and 20.6 for DEP) [8]. The LOD values of DAPs reported in our previous method [8] were decreased by half in the present method. The enhanced sensitivity is likely due to improved derivatization efficiency. While Timchalk et al. [25] adopted dehydration procedure for DAP measurement in rat biological sample, the present study is, to our knowledge, the first to indicate that the dehydration procedure prior to derivatization can improve sensitivity, accuracy and precision for the measurement of human urinary DAPs using liquid–liquid extraction and PFBBr derivatization. Moreover, the

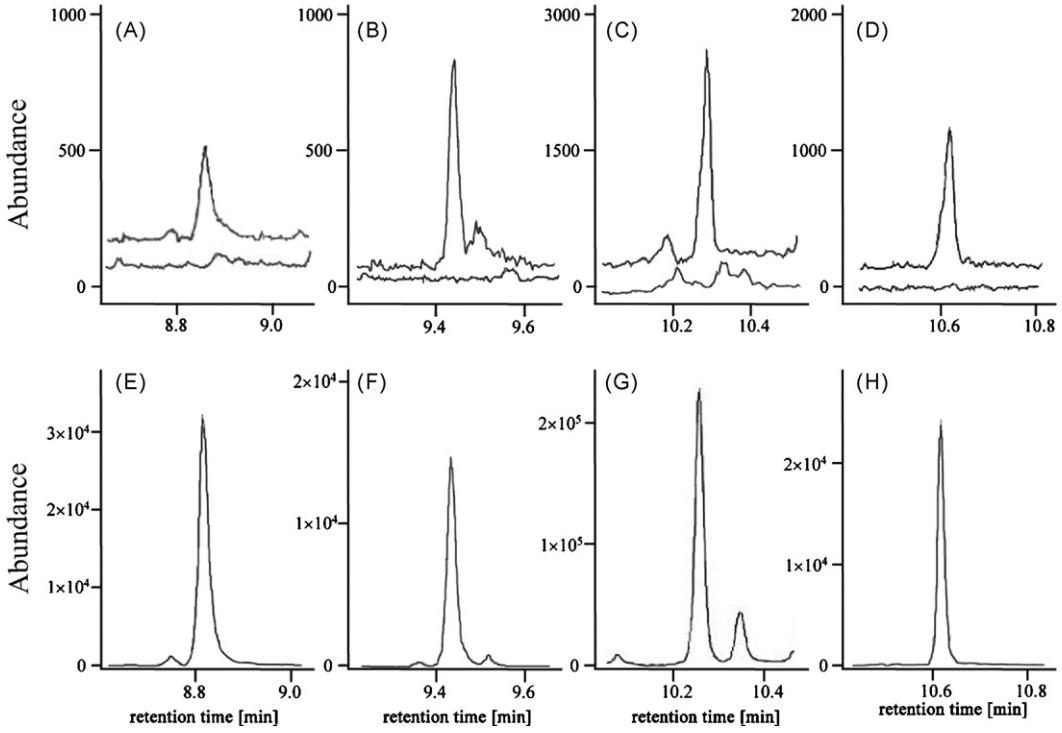


Fig. 2. Typical SIM GC–MS chromatograms in human urine samples under the LOD and near the LOQ level (A, DMP 1.0 μg/L; B, DEP 0.4 μg/L; C, DMTP 1.0 μg/L; D, DETP 0.2 μg/L), and DAP-spiked urine (E, DMP 47 μg/L; F, DEP 35 μg/L; G, DMTP 78 μg/L; H, DETP 22 μg/L). Detected masses for quantification were *m/z* 306 for DMP, 334 for DEP, 322 for DMTP, and 350 for DETP.

Table 3
Level of dialkyl phosphates in morning urine from healthy volunteers (control, $n = 25$) and persons engaged in pest control occupation (PCO, $n = 25$).

Dialkyl phosphates	Detected (%)	Geometric mean (μg/L of urine)	Median (μg/L of urine)	95th percentile (μg/L of urine)	Maximum (μg/L of urine)
DMP					
Controls	88	4.2	8.0	41.3	43.7
PCOs	100	70.0	39.1	765.9	862.0
DEP					
Controls	100	1.3	1.1	15.8	20.1
PCOs	100	7.1	5.8	92.3	106.8
DMTP					
Controls	100	4.2	3.9	40.7	45.9
PCOs	100	36.0	38.5	914.9	1044.2
DETP					
Controls	88	0.2	0.2	5.3	6.5
PCOs	100	0.9	0.7	26.1	28.0

clean-up procedure after derivatization using a multi-layer column effectively removed the highly irritating PFBBr and other interfering substances, resulting in a drastic reduction of unidentifiable peaks in both the total ion chromatogram (TIC) and the selected ion monitoring (SIM) chromatograms, and stabilization of the baseline.

Both GC–MS and LC–MS/MS methods have been developed to detect DAPs in urine [11,15,16], and these sophisticated analytical tools have inherent advantages in terms of high selectivity and sensitivity. The major advantage of the LC–MS/MS-based method is its ability to analyze various metabolites simultaneously with substantially simple sample pretreatment. However, these advantages are partially counterbalanced by the higher instrument cost in comparison with GC–MS systems. Moreover, sensitivity in DAP measurement using LC–MS/MS [15,16] at present tends to be lower than that using GC–MS or GC–MS/MS method [8,14,15]. In this study we combined the liquid–liquid extraction and GC–MS determination to achieve a lower-cost, faster and higher sample processing capacity.

3.3. Application of methods to field study samples

Fig. 2 shows the typical chromatogram in the SIM mode of under the LOD, near the LOQ and DAP spiked urine. Urinary concentration of DAPs in 25 controls and 25 PCOs are summarized in Table 3. DAPs were detected in 88–100% of all human samples. Geometric mean, median, 95th percentile and maximum levels of each DAP in the exposed group were obviously higher than those in the non-exposed one. The urinary DAP concentrations in the exposed workers are higher than those in our previous report [8]. The difference in the urine collection method may be the reason for this. Urine samples were collected on the next day after insecticide spraying in this study. But in our previous study, urine samples were collected when the workers underwent the health checkup. The urinary DAP concentrations in the non-exposed persons were approximately the same or less than those in previous reports [12,13,17,18].

One disadvantage in monitoring urinary DAP is the difficulty in estimating the OP exposure level using the measurement of DAPs from biological samples. DAP levels determined from biological sample might be affected by the intake of environmental DAP residue, thereby resulting in overestimation of the predicted dose of OPs. In fact, some researchers have reported the existence of DAPs in many foods [26]. Urinary DAP levels are usually reported as volume-weighted concentrations (e.g., μg/L) or creatinine-adjusted concentrations (e.g., μg/g creatinine). But the amount of daily creatinine excretion in urine varies according to age, sex, muscle mass and diet. Fortin et al. [27] suggested that the measurement of insecticide metabolite from spot urine samples may lead to serious errors in the estimation of the actual daily absorbed doses, even with adjustment of the creatinine contents. Further studies are needed to establish a feasible and scientifically acceptable data collection method to reflect the 24-h total excretion level.

Our method can be applied for DAP measurement in other biological samples such as blood, hair and amniotic fluid with slight modifications. Previously, Margariti et al. [28] determined hair DAPs using the same technique as ours with slight modifications.

4. Conclusion

Pre-derivatization process for effective anhydration, combined with a present GC–MS system, clearly improved sensitivity, precision, stability, and throughput without additional high-cost requirements. The present method would allow many laboratories to conduct the routine biological monitoring of urinary DAPs in the general population, and will be helpful in epidemiological study dealing with possible toxicity from low-level, long-term OP exposure.

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