

A revised method for determination of dialkylphosphate levels in human urine by solid-phase extraction and liquid chromatography with tandem mass spectrometry: application to human urine samples from Japanese children

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Abstract

Objectives Biological monitoring of organophosphorus insecticide (OP) metabolites, specifically dialkylphosphates (DAP) in urine, plays a key role in low-level exposure assessment of OP in individuals. The aims of this study are to develop a simple and sensitive method for determining four urinary DAPs using high-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS), and to assess the concentration range of urinary DAP in Japanese children.

Methods Deuterium-labeled DAPs were used as internal standards. Urinary dimethylphosphate (DMP) and diethylphosphate (DEP), which passed through the solid-phase extraction (SPE) column, and dimethylthiophosphate (DMTP) and diethylthiophosphate (DETP), which were extracted from a SPE column using 2.5 % NH₃ water including 50 % acetonitrile, were prepared for separation analysis. The samples were then injected into LC–MS/MS.

The optimized method was applied to spot urine samples from 3-year-old children (109 males and 116 females) living in Aichi Prefecture in Japan.

Results Results from the validation study demonstrated good within- and between-run precisions (<10.7 %) with low detection limits (0.4 for DMP and DMTP, 0.2 for DEP and 0.1 µg/L for DETP). The geometric mean values and detection rates of the urinary DAPs in Japanese children were 14.4 µg/L and 100 % for DMP, 5.3 µg/L and 98 % for DMTP, 5.5 µg/L and 99 % for DEP, and 0.6 µg/L and 80 % for DETP, respectively.

Conclusions The present high-throughput method is simple and reliable, and can thereby further contribute to development of an exposure assessment of OP. The present study is the first to reveal the DAP concentrations in young Japanese children.

Keywords Organophosphorus insecticide · Dialkylphosphate · Human urine sample · LC–MS/MS · Solid-phase extraction

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Introduction

Organophosphorus insecticides (OPs) have been indispensable, thanks to their low cost and beneficial effects on crop yield, forest protection and hygienic management in urban settings, despite the availability of newer insecticides such as pyrethroid and neonicotinoid insecticides [1]. Owing to the improved analytical methodology for urinary OP metabolites, dialkylphosphates (DAPs), they have been measured as a biomarker of OP for assessing not only occupational but low-dose environmental exposure. The measurement of urinary DAPs in epidemiological studies has gained in importance

since a variety of adverse health effects such as neurologic disorders and thyroid hormone disruption are associated with chronic low-dose exposures to OPs [2–5]. We have characterized urinary DAP concentrations in the Japanese with a previously used measurement method in three distinct occupational settings, i.e., a general working condition without occupational insecticide exposure, and indoor and outdoor pesticide spraying work conditions [6]. The results showed that urinary DAP concentrations in the general and occupationally OP-exposed populations were approximately at the same or lower levels compared with those measured in their overseas counterparts. The results also indicated that studies of the exposure assessment of OP specific to Japanese populations should be developed due to the diverse characteristics of OP exposure levels among countries. Although many researchers have recently investigated OP exposure risks in children, no basic information regarding urinary DAP concentrations in Japanese children has been available [6–9].

The difficulty in measuring urinary DAP due to its low-level concentration hinders further development of epidemiological studies dealing with the possible toxicity from low-level, long-term OP exposure. Urinary DAP measurement is composed mainly of two procedures, sample preparation steps and separation analysis. Some preparation techniques such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), lyophilization and azeotropic distillation have been reported (Table 1). SPE is superior to other extraction method in terms of its higher selectivity, easier and speedy handling, and reduction of hazardous extraction solvents. Gas chromatography (GC)-mass spectrometry (MS), GC-MS/MS, or high-performance liquid chromatography (LC)-MS/MS have been commonly-used apparatuses for the separation analysis. However, each of these separation techniques has its own merits and demerits. The notable demerits in GC-based methods are labor-intensive and time-consuming derivatization steps, and in LC-based methods, the demerits are relatively poor sensitivities. The drawbacks of the LC-based method can be mitigated when triethylamine (TEA) is used as an ion pair reagent and an ionization accelerator of DAPs [20, 21]. However, TEA has a high possibility of contaminating LC pipes, and thus the measurement in the positive ion mode might be interfered by an intense noise originating from such contamination [21]. To address this issue, the purpose of this study is to establish a simple and sensitive LC-MS/MS method without using TEA, for measurement of urinary DAPs, dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), and diethylthiophosphate (DETP), and to evaluate DAP concentrations in urine from Japanese children.

Table 1 Analytical methods for detecting dialkylphosphates in human urine samples

References	Sample preparation	Instruments for separation analysis
Nutley et al. [10]	Azeotropic distillation	GC-FPD
Aprea et al. [11]	Azeotropic distillation	GC-FPD
Moate et al. [12]	SPE	GC-FPD
Hardt et al. [13]	LLE	GC-MS
Oglopline et al. [14]	Lyophilization	GC-MS/MS
Lin et al. [15]	SPE	GC-FPD
Brabo et al. [16]	Azeotropic distillation	GC-MS/MS
Ueyama et al. [17]	LLE + SPE	GC-MS
De Alwis et al. [18]	SPE	GC-MS/MS
Dulaurent et al. [19]	LLE	LC-MS/MS
Odetokun et al. [20]	SPE	LC-MS/MS

SPE solid-phase extraction, LLE liquid–liquid extraction, GC gas chromatography, FPD flame photometric detector, LC liquid chromatography, MS mass spectrometry

Materials and methods

Chemicals and reagents

DMP tetramethylammonium salt (99.9 %), DMTP ammonium salt (98.9 %), DEP (98.2 %), DETP ammonium salt (95.2 %), DMP sodium salt-d₆ (98.7 %), DMTP potassium salt-d₆ (99.0 %), DEP ammonium salt-d₁₀ (99.2 %) and DETP potassium salt-d₁₀ (98.2 %) were purchased from Hayashi Pure Chemical Ind. (Osaka, Japan). Formic acid, acetonitrile, water, and ammonium formate (each LC-MS grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Special grade formic acid and 25 % ammonia solution were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich, respectively. All other reagents were of analytical grade. The Oasis WAX SPE column (Waters Corporation; Milford, MA, USA), which was packed with polymeric reversed-phase and weak anion exchange mixed-mode sorbent, was used for DAP extraction from urine samples.

Preparation of stock solutions

Standard DAPs were diluted to a concentration of 1,000 mg/L in acetonitrile, and further diluted with the same respective solvents to prepare working standard solutions at concentrations ranging from 0.1 to 10 mg/L. Deuterium-labeled DAPs, for use as internal standard (I.S.) substance, were diluted by acetonitrile to a concentration of 30 mg/L for DMP-d₆ and DMTP-d₆, and 5 mg/L for DEP-d₁₀ and DETP-d₁₀ (I.S. stock solution). These solutions were stored in the dark at –30 °C, and were used within 3 months. Urine, collected from three healthy volunteers

who were neither treated with drugs nor occupationally exposed to chemicals, was used in basic methodological studies such as validation assay, matrix-matched calibration, and quality control. DAP-spiked pooled urine samples were prepared by addition of working standard solution <1 % volume to the urine.

Sample preparation procedure

A flow chart of the determination procedure for urinary DAPs is shown in Fig. 1. One milliliter of urine was pipetted into a 10-mL screw-top glass test tube, and 1 mL distilled water, 20 μL formic acid (100 mmol/L) and 20 μL I.S. stock solution were added. After gentle shaking, the test tube was incubated at 37 °C in a water bath for 10 min to decrease urinary turbidity. Then, the sample was applied to the SPE procedure.

A SPE cartridge packed into a 1-mL solvent reservoir was pre-conditioned with 1 mL of 2.5 % NH₃ and acetonitrile mixture (1:1, v/v), followed by wash with 1 mL formic acid (1 mmol/L). Then, the conditioned SPE was loaded with a 1.8-mL urine sample (containing distilled water, I.S. solution and formic acid), and placed under

vacuum pressure. Passed samples were collected into new plastic tubes. After incubation (4 °C for 15 min) and centrifugation (10,000×g for 5 min at 4 °C), the supernatant was injected into the LC for DMP and DEP measurement. On the other hand, DMTP and DETP were extracted by continuing use of the SPE column through which DMP and DEP passed. After column wash with 1 mL acetonitrile, DETP and DMTP were eluted by 1 mL 2.5 % NH₃ and acetonitrile mixture (1:1, v/v). The eluate was dried up with a gentle nitrogen stream at 40 °C and the residue was resolved with 300 μL acetonitrile. After 30-min sonication in an ultrasonic bath and centrifugation (2,000×g for 5 min), the supernatant was injected into the LC for DMTP and DETP measurement.

Chromatography and mass spectrometry conditions

LC–MS/MS analysis was run on an Agilent 1260 Infinity Binary LC system coupled with an Agilent 6430 triple quadrupole mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA). The LC operating conditions were as follows: LC column, Scherzo SM-C18 (Imtakt, Kyoto, Japan), 100 × 2 mm i.d., 3 μm silica; mobile phase,

Fig. 1 Analytical procedure for urinary dialkylphosphates

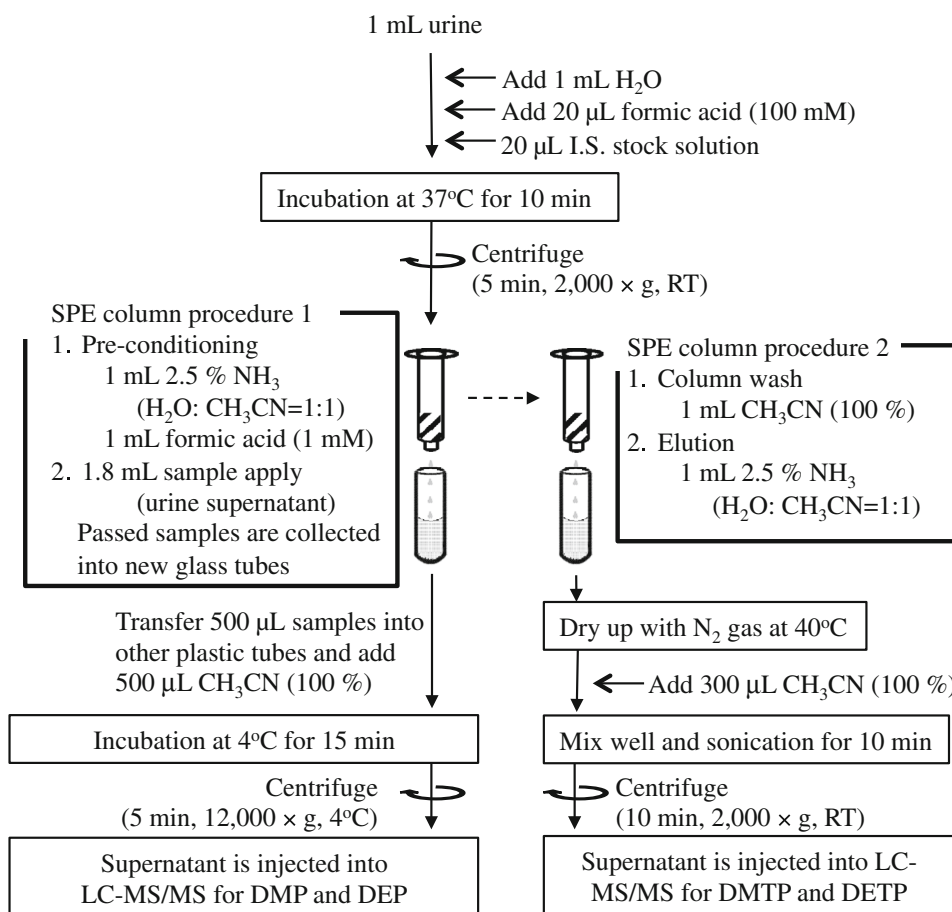


Table 2 Compound-specific mass spectrometer settings

Compounds	Fragmentor (V)	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (eV)	Polarity	Retention time (min)
DMP-d ₆	90	133	115	18	Positive	6.48
DMP	90	127	109 (Q) 95 (C)	20 20	Positive	6.48
DMTP-d ₆	90	147	129	14	Negative	6.54
DMTP	90	141	126 (Q) ^a 96 (C)	13 21	Negative	6.54
DEP-d ₁₀	80	165	101	16	Positive	6.53
DEP	80	155	127 (Q) 99 (C)	10 10	Positive	6.53
DETP-d ₁₀	100	179	95	20	Negative	6.89
DETP	90	169	95 (Q) 141 (C)	17 9	Negative	6.89

C confirmation ion,
Q quantification ion

^a Total of Q and C were used for DMTP quantification due to its low sensitivity

1 mmol/L formic acid and acetonitrile mixture (20:80, v/v) (A), and 10 mmol/L formic acid solution containing 10 mmol/L ammonium formate and acetonitrile mixture (20:80, v/v) (B); gradient condition of mobile phase B, 0 % (for 1 min)–14 %/min–70 % (for 2 min)–70 %/min–0 % (for 3 min); total flow rate of mobile phase A and B, 0.2 mL/min; total run time per sample, 12 min. All chromatographic solvents were degassed in a sonication bath for 20 min. The injection volume was 20 µL.

DAP detections were achieved by a triple quadrupole mass spectrometer equipped with an electro spray ionization (ESI) source using multiple reaction monitoring (MRM). Nebulizer gas (nitrogen) pressure was set at 35 psi with a source temperature of 325 °C, and the gas flow rate was 10 L/min. Capillary voltage was 4,000 V (positive mode for DMP and DEP, negative mode for DMTP and DETP). High-purity nitrogen gas was used as collision cell gas. Table 2 shows the optimized MRM parameters and retention times for OP metabolites and their I.S. The raw chromatograph and mass spectrogram data were processed with MassHunter Software Workstation (Agilent).

Assay validation

The intra- and inter-day precisions of our method were evaluated by parallel analytical runs performed on the same day or on seven consecutive days. To determine and calculate absolute recoveries, we spiked DAPs at two different stages in the DAP measurement procedure; i.e., in the beginning of the extraction procedure (urine sample) and prior to the injection procedure into LC–MS/MS. We compared the I.S. ratios obtained at these two stages. Matrix-matched calibration curves were represented by the analyte/I.S. peaks area ratio versus the concentrations of the calibration samples ranging from 1 to 200 µg/L for DMP and DEP, and from 0.1 to 200 µg/L for DEP and

DETP (each curve was drawn using five different concentrations). These DAP concentration ranges were determined based on the urinary DAP concentration ranges observed in Japanese people [6]. Indeed, these calibration curves are applicable to more than about 95 % of the Japanese general population [6]. The limits of detection (LOD) and limits of quantitation (LOQ) were calculated on the assumption of signal-to-noise ratios of 3 and 10, respectively. Dilution validation was evaluated using urinary samples at high DAP concentrations obtained from occupationally exposed workers. The urinary DAP concentrations were as follows: 216 µg/L for DMP, 110 µg/L for DMTP and 76 µg/L for DEP. Dilution validation of DETP could not be evaluated due to its relatively low concentration (maximum DETP concentration was 2.6 µg/L among available urine samples in this study).

Sample stability

Stability of the DAPs in human urine was determined using DAP-spiked urine samples at two concentrations (20 and 200 µg/L for DMP, 2 and 20 µg/L for DMTP, DEP and DETP). Urine samples in screw-top glass tubes were incubated at 4 or 37 °C for 24 h, and then urinary DAPs were measured. DAP peak areas of urine incubated for 24 h were compared with those of urine right before incubation. For the freeze and thaw stability test, urine samples were stored at –80 °C for 24 h and thawed with tap water for 10 min. After incubation at room temperature for 1 h, the urine samples were stored at –80 °C for 24 h again. I.S. ratios for DAP peak areas obtained from freeze and thaw-cycled urine were compared with those of urine which was not frozen at any point. Stability of prepared samples, which were injected into the LC, was also tested at a concentration of 5 µg/L DAPs. DAPs in prepared samples were analyzed immediately, and stored on the LC

auto sampler at 4 °C for 24 h or 5 days. DAP stability was assessed by comparing peak areas of DAP samples stored for 24 h or 5 days with those samples stored for 0 h.

Cross-validation of DAP analysis between previously reported method and present method

Thirty-five urine samples, which were obtained from occupationally OP-exposed group (pest control operators), have been measured with both our previous method [17] and the present method, because we have to evaluate the analytical comparability of these data. The characteristics of pest control operators were mentioned in our previous paper [6]. Pearson's correlation coefficient and intraclass correlation coefficient (two-way random model) (ICC) statistics were calculated after logarithmic transformation of urinary DAP concentrations. Statistical analysis was performed with SPSS® 19.0 (SPSS Inc., Chicago, IL, USA).

Urinary DAPs in young Japanese children aged 3 years

The study population consisted of 3-year-old children who lived in Inuyama and Okazaki City, Aichi Prefecture (central area of Japan) between August 2012 and February 2013. Informed consent forms and questionnaires were mailed to 708 families who had 3-year-old children scheduled to participate in a health checkup for 3-year-olds conducted by the city governments. Prior to enrollment in the study, parents of each subjects signed an informed consent form permitting the use of personal information and urine for research purposes. Four hundred and four subjects were recruited for this study, and their questionnaires and urine samples were obtained. Collected urine samples were transported at 4 °C to our laboratory, and then stored at –80 °C until DAP analyses. Eventually, 225 morning urine samples were obtained from the subjects, each donating more than 4 mL of urine, and they were applied for our methods. DAP concentrations of these samples may well be representative data of children who live in the area. The Ethics Committee of Nagoya University Graduate School of Medicine approved the study protocol, along with the Helsinki Declaration. Urinary creatinine concentrations were measured according to the previously reported method using LC [22].

Results

Optimization of sample preparation and LC–MS/MS conditions

SPE procedure for DAP extraction was adopted in this study to assure easy handling and high purification. As

shown in Fig. 1, we devised a new method that allows DMP and DEP in urine to pass through weak anion exchange SPE columns. Mass chromatogram of pooled urine spiked with DAPs at concentration ranges from 0 to 200 µg/L is shown in Fig. 2.

Assay validation

The accuracy, precision, and linearity parameters are summarized in Table 3. The DMP recoveries by this method yielded 95 and 100 % concentrations in 1 and 5 µg/L of urine, respectively. For the within-run precision, the percent of relative standard deviation (%RSD) ranged from 2.7 to 10.7 %. For the between-run precision, the %RSD was between 4.3 and 9.6 %. These precision data improved considerably in comparison with our previous method [17]. Reproducibility deteriorated when the I.S was not used. Calibration curves were constructed by plotting the peak area ratio of DAPs to I.S. (*y* axis) versus the concentration of DAPs (*x* axis). The regression equations were $y = 0.446x - 0.84$ for DMP, $y = 0.024x + 0.15$ for DMTP, $y = 0.073x - 0.16$ for DEP, and $y = 0.060x + 0.035$ for DETP. A good dilution validation was confirmed when serially diluted urine samples were analyzed between the dilution ratio and analyte/I.S ratio ($r^2 > 0.9997$ for DMP, DMTP and DEP), suggesting that urine matrix had no effect on ionization of DMP, DMTP and DEP.

Sample stability

The stabilities of DAPs in urine and prepared samples were assessed under typical storage/handling conditions (Table 4). Incubation of urine samples at 4 or 37 °C caused negligible changes in DAP peak areas (93–112 % for urinary DAPs). DAP peak-areas in four-time freeze and thaw-cycled urine were approximately at the same level as those in fresh urine samples (94–111 % for urinary DAPs).

Comparison of DAP concentrations measured by two methods

We evaluated whether there were comparability of DAP data obtained from previous method (GC–MS) and the present method (LC–MS/MS). Geometric means of urinary DAP concentrations in pest control operators measured by the present method (LC–MS/MS) were 8.9 µg/L (max. 256.4 µg/L) for DMP, 3.5 µg/L (max. 81.9 µg/L) for DMTP, 2.9 µg/L (max. 88.2 µg/L) for DEP and 0.4 µg/L (max. 5.7 µg/L) for DETP. Pearson's correlation coefficient and ICC were as follows: DMP, 0.829 and 0.747; DMTP, 0.843 and 0.740; DEP, 0.766 and 0.616; DETP, 0.831 and 0.532.

Fig. 2 Mass chromatogram of dialkylphosphates at concentration range from 0 to 200 µg/L

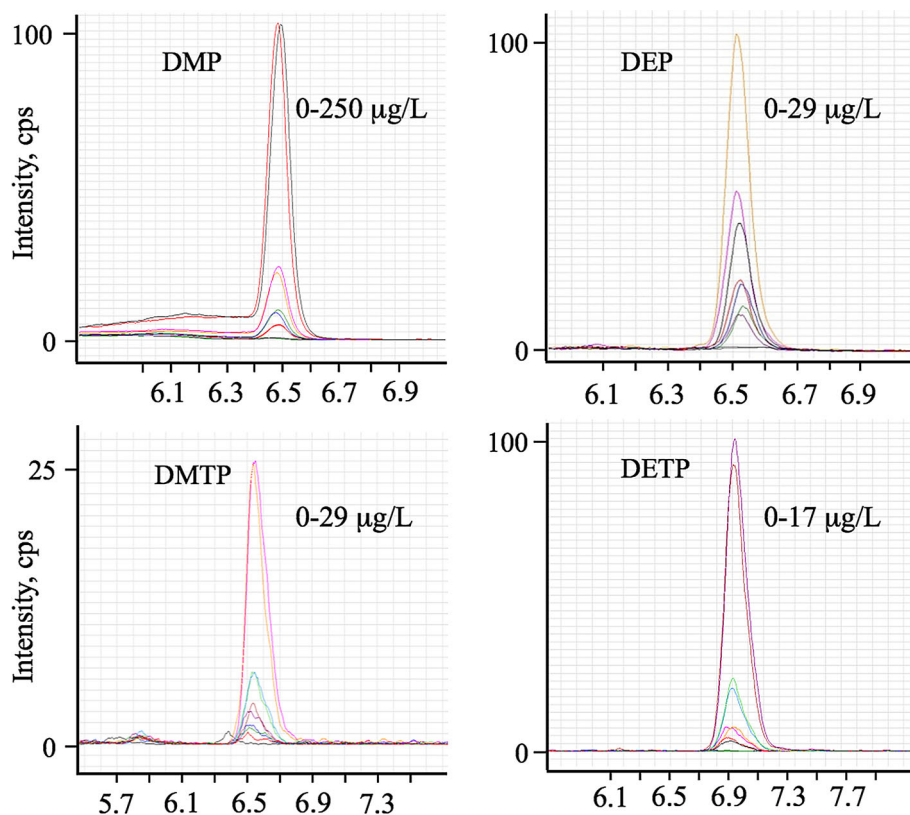


Table 3 Accuracy, precision, LOD and LOQ data of analytical procedure

	Concentration (µg/L)	<i>n</i>	DMP	DMTP	DEP	DETP
Within-run precision (%RSD)	1	4			10.3	6.4
	2	4	3.1	9.8	10.7	3.4
	5	4	3.1	2.7	3.9	4.5
	10	4	3.6	5.8		
	20	4			5.5	3.2
	100	4	6.7	3.6		
Mean recovery ^a	1	2	95	64	81	101
	5	2	100	105	82	93
Between-run precision (%RSD)	1	7	6.9	7.7	9.6	7.6
	10	7	9.0	4.3	7.5	7.8
<i>R</i> ² of calibration line			0.999	0.998	0.997	0.999
LOD (µg/L) (S/N = 3)			0.4	0.4	0.2	0.1
LOQ (µg/L) (S/N = 10)			1.2	1.2	0.6	0.3

n number of observations, *RSD* relative standard deviation, *LOD* limit of detection, *LOQ* limit of quantitation

^a Recovery obtained by adding the standards just before the separation analysis step

Table 4 Storage stability of urinary DAPs

Condition	Concentration (µg/L urine)	%RSD			
		DMP	DMTP	DEP	DETP
Urine sample					
Stored at 4 °C	2		111	94	112
	20	93	101	102	93
	200	99			
Stored at 37 °C	2		106	109	108
	20	97	104	104	102
	200	100			
After four times freeze–thaw cycles					
	2		97	94	96
	20	95	100	104	103
	200	111			
Prepared sample					
Stored at 4 °C for 1 day	5	93	97	96	95
	5	90	109	104	102

RSD relative standard deviation

Application for environmental OP exposures

Another purpose of this study is to evaluate DAP concentrations in Japanese children. The detection rates of

Table 5 Urinary DAP concentrations reported in previous papers

Author Sampling country	Study population (years)	Units	Sample size	GM				95 percentile			
				DMP	DMTP	DEP	DETP	DMP	DMTP	DEP	DETP
Barr et al. [7], USA (NHANES 2003–2004)	6–11	µg/L	296	NC	2.8	NC	NC	16.2	30.9	16.1	2.2
		µg/g cre	–310	NC	3.4	NC	NC	19.6	36.1	16.1	2.7
Becker et al. [8], Germany	2–17	µg/L	363	13.5	8.2	3.3	1.1	118	124	20.0	11.0
		µg/g cre		10.7	6.5	2.6	0.9	95.8	109	14.1	-
Babina et al. [9] ^a , Australia (Periurban area)	2.5–6	µg/L	111	–	–	–	–	–	–	–	–
		µg/g cre		–	24.8	15.4	7.9	–	698	387	114
Present study, Japan	3	µg/L	225	14.4	5.3	5.5	0.6	59.0	65.3	25.2	5.0
		µg/g cre		21.0	7.8	8.0	0.9	68.0	81.7	33.1	6.3

NC not calculated, NHANES National Health and Nutrition Examination Survey, GM geometric mean, cre creatinine

^a Median values were represented instead of GM

urinary DAPs were 80–100 %. The geometric means and 95 percentile were shown in Table 5. This implies that our method can be applied to the biomonitoring of urinary DAPs of Japanese children who are environmentally exposed to OP. We confirmed that the distribution of concentrations of urinary DAPs was normalized by logarithmic transformation.

Discussion

First of all, an optimum sample preparation method was sought so as to yield a sensitive determination. Dulaurent et al. [19] adopted liquid–liquid DAP extraction using diethylether and ethylacetate, and obtained low recoveries (13 % for DMP and 25 % for DMTP) at a DAP concentration of 2 µg/L. Odetokun et al. [20] selected weak anion exchange SPE columns (Strata X-AW, Phenomenex Inc; Torrance, CA, USA) as the DAP extraction column with a DMP recovery rate of only 40 %. These low recoveries of DMP were due in part to the high polarity and weak positive charge of DMP compared with other DAPs. Therefore, the high absolute recovery of DAPs in our method can be highlighted as significant. Although recovery rate of DEP at concentration of 1 µg/L was relatively low, the extent of recoveries of analytes was reproducible.

The analytical separation of the four analytes in this study was performed using a ODS column consisting of C18, weak anion, and weak cation ligands. This hybrid column allowed DAP separation from urine matrix without addition of TEA as an ion pair reagent. Moreover, injection of a large volume of prepared samples (20 µL) into the LC contributed to highly sensitive DAP detection without TEA addition as an accelerating reagent for DAP ionization. This may have been caused by the condensation of DAPs at

the forefront of the analytical column after the sample injection into the LC, because the weak anion exchange ligand can hold DAPs tightly under a condition of abundant mobile phase A. The retention times of DAPs were similar to each other. We have confirmed that the concentration of one DAP component was not affected by that of the other components.

Although dibutylphosphate was used as the I.S. in our previous studies using GC–MS [11], the present method did not show good within-run precision when using dibutylphosphate (%RSD >15 %), suggesting that deuterium-labeled DAPs are indispensable when using LC–MS/MS to obtain ideal within-run precision.

Odetokun et al. [20] and we [17] have reported a DAP measurement method with sufficient LODs of 0.5 and 0.3 µg/L for DMP, 0.07 and 0.1 µg/L for DMTP, 0.05 and 0.1 µg/L for DEP and 0.11 and 0.1 µg/L for DETP. Although the LODs for DAPs in this study are not lower than those in Odetokun’s study, the LODs in ours are sufficiently low for measurement of each urinary DAP from the Japanese general population [6]. It can be presumed that the high DAP recoveries and the large volume sample injection into the LC–MS/MS achieved highly sensitive analyses without TEA.

The results of sample stability examination corroborate that the stability of urinary DAPs is unaffected by the usual collecting conditions in the field survey. Further studies concerning DAP stability using urine samples obtained from individuals under various medical conditions are needed, because it is not clear how the variability of urine components such as oxidants and drugs in urine affects DAP stability.

We previously demonstrated that the geometric means of summertime urinary DMP, DMTP, DEP and DETP concentrations in 164 Japanese adults (42 ± 10 years, mean ± SD) were 10.3, 5.1, 1.3 and 0.4 µg/L, respectively

(7.0, 3.4, 0.8 and 0.3 $\mu\text{g/g}$ creatinine, respectively) [6]. These data were obtained using our previous method using GC–MS [17]. From a statistical viewpoint [23], more than 0.81 of ICC are categorized as ‘almost perfect’. The ICC level 0.61–0.81 and 0.41–0.60 are categorized as ‘substantial’ and ‘moderate’, respectively. Given the high Pearson’s correlation and ICC, the DAP concentrations measured by our previous method [17] and present method may be reliably comparable, except for DETP due to its low concentration. To ameliorate the ICC of DETP, we might have to adopt duplicate or triplicate assay.

Although urinary DAP concentrations in Japanese children are seemingly higher than those in Japanese adults, careful considerations should be made about the differences in physiologic characteristics between adults and children, such as OP metabolizing enzyme activity, urine volume, and urinary creatinine excretion level. In an international comparison of the geometric mean or median level of urinary DAP concentrations, it is likely that the DMP concentration in our data was approximately the same or higher than that of US (6–11 years of age) [7], German (2–17 years of age) [8] and Australian (2.5–6 years of age) [9] children (Table 5). Urine volume and creatinine excretion in urine increase with increasing age toward adults. An earlier study by Babina et al. [9] has suggested that urinary DAP concentrations in urban area children were higher than those in rural area children. Moreover, we reported that total DAP concentrations in summer were higher than those in winter [6]. Therefore, it is difficult at present to conclude that higher OP exposures have occurred in Japanese children than in other countries.

A further study of biological monitoring of urinary DAPs from a large number of subjects would be needed to evaluate the adverse effects of OP exposure. Since our simple and sensitive method for DAP determination can also achieve a high-throughput measurement without labor-intensive operation, it can be applied to routine biological monitoring of urinary DAPs, not only in occupational health studies but also in epidemiological studies.

In conclusion, a sensitive and reliable LC–MS/MS method with SPE sample preparation for the determination of urinary DAPs was developed. The main strengths of the present study are its development of a TEA-free sensitive determination of urinary DAPs, and its first report about urinary DAP concentrations in Japanese children. This study is a major step toward the human risk assessment of OP exposures.

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Conflict of interest The authors declare that they have no conflict of interest.

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