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Determination of Five Phthalate Monoesters in Human Urine Using Gas Chromatography-Mass Spectrometry

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Abstract We have developed a gas chromatography-mass spectrometry (GC–MS) method to determine five phthalate monoesters (monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-(2-ethylhexyl) phthalate (MEHP), monoisononyl phthalate (MINP) and monobenzyl phthalate (MBz)) in human urine. Human urine samples were subjected to enzymatic deconjugation of the glucuronides followed by extraction with hexane. The extracted phthalate monoesters were methylated with diazomethane, purified on a Florisil column and then subjected to GC–MS analysis. The recoveries from urine spiked with five phthalate monoesters were 86.3%–119% with coefficients of variation of 0.6%–6.1%. We measured phthalate monoester levels in human urine by analyzing 36 samples from volunteers. MBP and MEP were detected in all samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINPs were not detected in most samples (6% detectable). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP than men. The estimated daily exposure levels for the four parent phthalates excluding diisononyl phthalate ranged from 0.27 to 5.69 μg/kg/day (median).

Keywords Phthalate monoesters · Exposure · Urine · Gas chromatography-mass spectrometry

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Phthalates, diesters of phthalic acid, are widely used in many consumer products and as additives, solvents and plasticizers. Humans are ubiquitously exposed to phthalates (Blount et al. 2000a; Silva et al. 2004) and patients undergoing medical procedures such as transfusions, dialysis, cardiopulmonary bypass or aphaeresis are potentially more heavily exposed (Calafat et al. 2004; Green et al. 2005; Inoue et al. 2005). Certain phthalates such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP) lead to testicular toxicity in rodents in an antiandrogenic manner (Parks et al. 2000). DBP, DEHP, BBzP, and their metabolites, mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and mono-(2-ethylhexyl) phthalate (MEHP) are teratogenic in animals (Gray and Gangolli 1986; Mylchreest et al. 1998; Parks et al. 2000; Ema and Miyawaki 2001a, b). It has been reported that urinary concentrations of four phthalate metabolites, monoethyl phthalate (MEP), MBP, MBzP, monoisobutyl phthalate, were significantly associated with reduced anogenital distance in human male infants which was the first

demonstration of the subtle developmental effects in human (Swan et al. 2005).

More recent studies concerning general human exposure to phthalates have been carried out by measuring phthalate monoester metabolites mainly in urine (Koch et al. 2003; Hauser 2008), because phthalate diesters metabolize rapidly to their monoesters and are excreted in urine either as hydrolytic monoesters or the oxidative products (Silva et al. 2003). Furthermore, potential problems of diester contamination during sample collection, storage and analysis procedures may make it difficult to measure low baseline levels of phthalate diesters in serum and/or urine (Blount et al. 2000b). Several reports have described the assessment of phthalate exposure in the general population in the USA (Kohn et al. 2000) and Europe (Koch et al. 2003). However, reports relating to the Japanese are limited; Itoh et al. (2005) measured MBP and MEHP in urine samples from the general population.

For the quantitative detection of phthalate monoester metabolites in urine, a high-performance liquid chromatography-tandem mass spectrometric (LC–MS/MS) method has been developed (Silva et al. 2003). All the above-mentioned reports used LC–MS/MS method. To our knowledge, there are no reports of studies on phthalate monoester determination in urine using gas chromatography-mass spectrometry (GC–MS). In the present study we have developed a new method for the quantitative determination of five phthalate monoesters (MEP, MBP, MEHP, monoisononyl phthalate (MINP) and MBzP) in human urine using GC–MS. The method was used for analysis of human urine samples.

Materials and Methods

The study participants were 36 volunteers who were recruited from the staff of Aichi Prefectural Institute of Public Health, Nagoya, Japan. All of the volunteers reside in the Aichi Prefecture, Japan. Of the participants, 64% (23/36) were men and 36% (13/36) were women. 31% (11/36) were under 39 years of age, 25% (9/36) were between 40 and 49 years of age, and 44% (16/36) were over 50 years of age.

MEP, MBP, mono-2-ethylhexyl phthalate (MEHP), monoisononyl phthalate (MINP), MBzP, and their $^{13}C_4$-labeled internal standards were purchased from Cambridge Isotope Laboratories (MA, USA). Florisil® PR, β-glucuronidase solution from *Escherichia coli* and pesticide analysis grade sodium sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 μm pore size) was purchased from Varian (CA, USA). Phthalic acid esters, analysis grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical (Tokyo, Japan). The water used for the extraction was prepared by washing distilled water with hexane.

All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil® PR were heated at 200°C for 2 h. A reagent blank was analyzed before sample analysis in each batch.

A Florisil column was prepared by packing Florisil (1 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm × 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Diazomethane was prepared by adding 0.5 g of *N*-methyl-*N*′-nitro-*N*- nitrosoguanidine to 3 mL of 20% sodium hydride solution; the diazomethane gas thus generated was dissolved in 10 mL of ice-cold methyl *t*-butyl ether.

Urine (2 mL) was placed into a centrifuge tube to which ammonium acetate (0.5 mL) was added and the sample was spiked with isotopically labeled internal standards (50 μL, 1 μg/mL). After β-glucuronidase (30 μL, 50 units/mL) was added, the sample was incubated at 37°C for 60 min. The sample solution was then acidified to pH 2 with 10% sulfuric acid. After adding hexane (5 mL) and sodium chloride (1 g), the sample solution was mixed for 3 min by vigorous shaking and then centrifuged at 3,000 rpm for 5 min. The hexane layer was collected and dried under a stream of nitrogen gas at 35°C. Diazomethane (0.5 mL) was added to the residue and the mixture was allowed to stand for 30 min at room temperature. The solution was dried under a stream of nitrogen gas at 35°C. The residue was dissolved in hexane (5 mL) and then loaded onto a Florisil column, which had been preconditioned with acetone (10 mL) and hexane (10 mL). After washing with hexane (3 mL), the phthalate monoester fraction was eluted with 5% acetone-hexane (10 mL). The eluate was concentrated to 1.0 mL under a stream of nitrogen gas at 35°C. An aliquot of each sample (2 μL) was injected into a GC–MS system.

GC–MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30-m HP-5MS SV column (J & W Scientific, CA, USA) of i.d. 0.25 mm and a film thickness of 0.5 μm was used. The initial oven temperature was 80°C. After holding at the initial temperature for 3 min, the temperature was increased to 240°C at a rate of 20°C/min, and then to 300°C at a rate of 10 °C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min. The ion source temperature was 230°C and electron ionization was used as the ionization mode. The injection port was kept at 250°C. The ions used for selected ion monitoring (SIM) are summarized in Table 1; the ions observed as the base peak were used for quantification and

Table 1 Monitored ions for quantification and confirmation, and retention times for the measured methyl derivatives of phthalate monoesters

Analyte	Monitored ions (*m/z*)		Retention time (min)
	Quantification	Confirmation	
MEP	163	149, 176	9.04
MEP-$^{13}C_4$	167	153, 180	9.04
MBP	163	149, 181	10.18
MBP-$^{13}C_4$	167	153,185	10.18
MEHP	163	149, 181	12.09
MEHP-^{13}C4	167	153,185	12.09
MINP	163	149, 181	12.42
MINP-$^{13}C_4$	167	153,185	12.42
MBzP	163	91, 164	12.71
MBzP-^{13}C4	167	91, 168	12.71

Abbreviations: *MEP* monoethyl phthalate, *MBP* mono-*n*-butyl phthalate, *MEHP* mono-2-ethylhexyl phthalate, *MINP* monoisononyl phthalate, *MBzP* monobenzyl phthalate

the second and third most abundant ions were used for confirmation. The concentrations of five phthalate monoesters in urine were corrected with $^{13}C_4$-labeled internal standards. Reproducible calibration curves for the methyl derivatives of the five phthalate monoesters were obtained with correlation coefficients greater than 0.999 (known concentration versus analyte/internal standard) and they were linear over the range of 2–100 ng/mL.

We calculated the estimated daily intake of phthalates by using the following equation (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005): intake (μg/kg/day) = ME × CE/(f × 1,000) × (MW_d/MW_m). ME is the creatinine-adjusted concentration of the phthalate monoester (μg/g creatinine), CE is the personal daily creatinine excretion (mg/kg/day), which was calculated using the following equation (Kawasaki et al. 1991): CE (male) = − 12.63 × age + 15.12 × body weight (kg) + 7.39 × height (cm) − 79.90, or CE (female) = −4.72 × age + 8.58 × body weight (kg) + 5.09 × height (cm) − 74.95. The values of the ratios of urinary excretion to total elimination (f) were 0.024 for DEHP, 0.69 for DEP and DBP, and 0.73 for BBzP (Koch et al. 2003). MW_d and MW_m are the molecular weights of the parent diesters and their monoesters, respectively.

The Mann–Whitney *U*-test was used for statistical analyses. Differences were considered to be significant at $p < 0.05$.

Results and Discussion

For extraction of phthalate monoesters in biological samples, different solid-phase extraction (SPE) and solvent extraction methods are used. Among a variety of commercially available solid-phase extraction cartridges, we examined three types of cartridge; Abselut NEXUS (VARIAN), GL-Pak Glass SPE PLS-3 (GL Science, Tokyo, Japan) and Oasis® HLB Glass (Waters, MA, USA). However, the background levels of MEHP and MBP for all cartridges were high at about 60 and 8 ng/mL, respectively, even though the cartridges were prewashed with acetone and hexane repeatedly. We then examined hexane and acetonitrile for extraction of phthalate monoesters. Although both solvents extracted phthalate monoesters in urine effectively, acetonitrile also extracted co-existing substances in urine. Hexane was consequently selected as the extraction solvent.

For purification of methyl derivatives of phthalate monoesters methylated with diazomethane, we examined the Florisil single layer column and the Florisil and Bondesil-PSA dual layer column. A Florisil single layer column was selected because both columns showed the same clean-up effect. For elution from a Florisil column, we examined a mixture of solvents; acetonitrile/hexane and acetone/hexane. Acetone/hexane was selected because this solvent mixture gave the most satisfactory recoveries, while acetonitrile/hexane gave poor recoveries of methylated MEP (<10%). The optimized procedures including the extraction followed by Florisil column purification are described in the Materials and Methods section. Typical SIM chromatograms of a mixture of methyl derivatives of five standard phthalate monoesters and a urine sample are shown in Fig. 1.

The recoveries from urine spiked with 250 ng/mL of each of the five phthalate monoesters were examined by calculating the ratio of the amount of analytes recovered

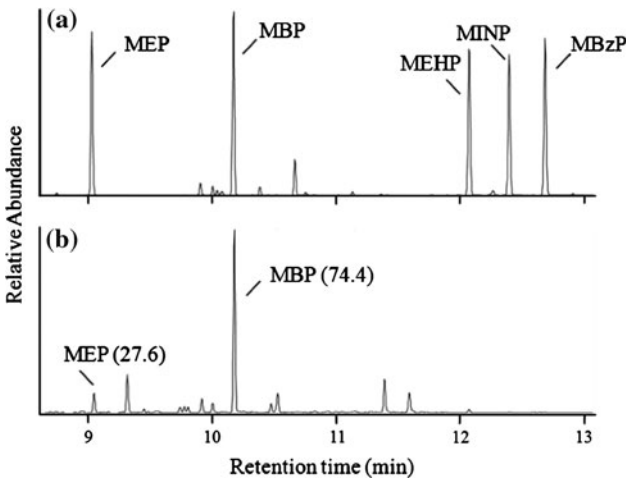

Fig. 1 Typical SIM chromatograms of **a** a mixture of methyl derivatives of five standard phthalate monoesters and **b** a urine sample. *Numbers in parentheses* indicate the found concentrations (ng/mL)

Table 2 Recoveries and LOQ of phthalate monoesters

Analyte (ng/mL)	Recovery (%)	CV[a] (%)	LOQ[b]
MEP	86.3	2.2	1
MBP	119	6.1	1
MEHP	100	0.6	5
MINP	98.0	0.8	1
MBzP	89.1	2.1	5

Results are means of three replicate determinations

[a] *CV* coefficients of variation

[b] *LOQ* limit of quantification

after Florisil column purification to the amounts originally added. Overall recoveries and coefficients of variation were found to be satisfactory; these values were 86.3%–119% and 0.6%–6.1%, respectively (Table 2). To determine the background levels of the five phthalate monoesters originating from sample preparation, a blank test was carried out using hexane-washed water instead of urine. The blanks ($n = 3$) contained < 1.0 ng/mL for MBP, 1.6 (± 0.09) ng/mL for MEP, 3.0 (± 0.5) ng/mL for MEHP, <1.0 ng/mL for MINP and <5.0 ng/mL for MBzP. The limit of quantification (LOQ) for each of the five phthalate monoesters was calculated as $10S_0$, where S_0 is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as $10S_1$, where S_1 is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of the standard sample. The LOQ for each of the five phthalate monoesters is summarized in Table 2. With careful control of contamination, as described in the Materials and Methods section, sample analysis was achieved with a low-level background, which allowed us to evaluate precisely the concentration of phthalate monoesters.

This method was then applied to the analysis of urine samples obtained from 36 volunteers (Table 3). MBP and MEP were detected in all samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINP was not detected in most samples (detectable in 6%). The concentrations of MBP and MEP showed larger inter-individual variation: 9.09–194 ng/mL for MBP and 1.36–1,350 ng/mL for MEP, whereas those of MBzP, MEHP and MINP showed less inter-individual variation; <LOQ-40.6 ng/mL for MBzP, <LOQ-29.6 ng/mL for MEHP and <LOQ-3.00 ng/mL for MINP. We speculate that these results may reflect the differences in the individuals' use of products containing the parent phthalate diesters. DBP and diethyl phthalate (DEP) are used mostly in consumer products (detergents, soaps, cosmetics, shampoo, and perfumes), while BBzP, DEHP and DINP are used primarily for industrial purposes as plasticizers (Silva et al. 2004; Mortensen et al. 2005). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP (114 and 110 μg/g creatinine) than did men (58.7 and 12.6 μg/g creatinine). The fact that women had higher concentrations of MBP and MEP than men was most likely attributable to women's increased use of personal care products, such as hair care products, cosmetics, and perfumes as indicated by Silva et al. (2004). Urinary levels of phthalate monoesters in approximately 2,540 samples collected from participants in the National Health and Nutrition Examination Survey (NHANES), 1999–2000 in the USA have been reported (Silva et al. 2004). The median concentration of MBP and MEHP was 2.7- and 2.5-fold lower in the NHANES 1999–2000 population (21.9 and 3.08 μg/g creatinine) than in the present study (58.7 and 7.73 μg/g creatinine), whereas that of MEP in the NHANES 1999–2000 population (141 μg/g creatinine) was almost tenfold higher than that in the present study (13.7 μg/g creatinine). The median concentrations of MBzP were almost the same in both studies (13.3 μg/g creatinine for NHANES 1999–2000 population and 11.4 μg/g creatinine for the present study). Despite the limited numbers of samples in the present study, these results may represent the differences in the use of phthalate diesters between the USA and Japan.

Human exposure to phthalate diesters has been estimated by calculating the daily intake using the concentrations of the phthalate monoesters in urine (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005). Estimates of the intake of four phthalate diesters excluding MINP are shown

Table 3 Concentrations of phthalate monoesters in urine

Analyte	No. of Positives	Concentration			
		Measured(ng/mL)		Creatinine-adjusted (μg/g creatinine)	
		Median	Range	Median	Range
MBP	36 (100)[a]	60.0	9.09–194	58.7	22.8–554
MEP	36 (100)	10.7	1.36–1,350	13.7	3.06–944
MBzP	27 (75)	10.9	<LOQ–40.6	11.4	<LOQ–39.4
MEHP	20 (56)	5.75	<LOQ-29.6	7.76	<LOQ-56.2
MINP	2 (6)	<LOQ	<LOQ-3.00	<LOQ	<LOQ-6.38

[a] Numbers in parentheses indicate percentages

Table 4 Comparison of the intake estimates for four phthalate diesters

Phthalate diester	Median intake value (μg/kg/day)				TDI (μg/kg/day)
	This study (n = 36)	Kohn et al. (n = 289)	Koch et al. (n = 85)	Itoh et al. (n = 36)	
DEHP	5.69	0.71 (3.14)[a]	10.3	1.8 (5.48)	40–140
DBP	1.47	1.5	5.22	1.3	100
DEP	0.29	12	2.32	–	5,000
BBzP	0.27	0.88	0.60	–	200

[a] Numbers in parentheses indicate the recalculated intake value by using the fractionary urinary excretion factor of 0.024 reported by Koch et al. (2003)

in Table 4 together with other previous assessments. The median values in the present study were highest for DEHP intake and decreased in the order DEHP, DBP, DEP, and BBzP. The median values of DEHP intake were higher in the present study (5.69 μg/kg/day) than those reported by Kohn et al. (2000) (0.71 μg/kg/day) and Itoh et al. (2005) (1.8 μg/kg/day), and were lower than those reported by Koch et al. (2003) (10.3 μg/kg/day). However, previous assessments used different fractional excretion values, suggesting that the calculated daily intake values have inherent uncertainties. When DEHP intake values are recalculated using the fractional urinary excretion factor (0.024) reported by Koch et al. (2003), these values range from 3.14 to 10.3 μg/kg/day (Table 4) which are comparatively similar values. The establishment of a method for calculating the intake estimates remains to be elucidated.

In summary, we have developed a gas chromatography–mass spectrometry method to determine the concentration of five phthalate monoesters in human urine. Using this method we were able to measure precisely the level of phthalate monoesters in human urine obtained from 36 volunteers. This method will help to evaluate human exposure to phthalates.

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