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Determination of total phthalates in urine by isotope-dilution liquid chromatography–tandem mass spectrometry

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Abstract

Diesters of 1,2-benzenedicarboxylic acid are a family of industrial compounds called "phthalates." The physical and chemical properties of these diesters, and therefore their potential uses, depend on the structure of the dialkyl or alkyl/aryl side chain. The urinary concentrations of phthalate monoesters, which are metabolites, have been used as biomarkers of human exposure to specific phthalates. However, several phthalates, particularly those with side chains of eight or more carbon atoms, are complex mixtures of isomers. For these, the phthalate metabolites to be used as biomarkers of exposure have not been unequivocally identified. We developed a method for assessing total exposure to phthalates, including the isomeric mixtures of high molecular weight phthalates, by measuring the concentration of phthalic acid (PA) in human urine after acid hydrolysis of the phthalate metabolites to PA. The present method accurately assesses total exposure to phthalates without noticeable contamination from the ubiquitous phthalates in the environment, but it gives no information about the parent phthalate.

Keywords: Phthalic acid; Acid hydrolysis; Phthalates; Total exposure; Body burden

1. Introduction

Phthalates are a family of industrial compounds with a common chemical structure—dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid—and different physical and chemical properties depending on the nature of the alkyl/aryl side chain. Phthalates have a wide spectrum of industrial applications, including their use as plasticizers of polyvinyl chloride and cellulose acetate; additives in pharmaceuticals, consumer and personal-care products; and lubricants [1–5].

Some phthalates are rodent carcinogens [4,6]; the relevance of carcinogenicity in humans is debatable [7,8]. Several phthalates and their metabolic products are reproductive toxicants affecting particularly male reproductive development in experimental animals [9–12]. Human studies are scarce, but exposure to some phthalates in adults has been associated with altered sperm and semen properties [13–15] and with altered pulmonary function [16].

Because of the high potential for human exposure and their demonstrated toxicity in animals, phthalates are a concern in the field of environmental public health. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate, can be further metabolized to oxidative products. Monoesters and the oxidative metabolites of phthalates may be conjugated as the glucuronide [1–4], and both free and conjugated metabolites can be excreted in the urine and feces. Phthalate monoester metabolites in urine have been used as markers to assess phthalate exposure to the general population [17–21] and to specific population groups [13–15,22–25].

Several high molecular weight phthalates (e.g., di-isooctyl phthalate (DiOP), di-isononyl phthalate (DiNP), di-isodecyl phthalate (DiDP)) are complex substances consisting of mixtures of isomers [5]. Subsequently, several metabolite isomers and their oxidative products may be expected for each phthalate, but most of these metabolites have not been un-

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equivocally identified. Therefore, some of the isomeric phthalates lack specific biomarkers of exposure or in the case a biomarker exists (e.g., mono-3-methyl-5-dimethylhexyl phthalate, a metabolite of DiNP), it reflects exposure to only one of the phthalate isomers, thus underestimating the actual exposure to the isomeric mixture. Moreover, for the long-alkyl chain phthalates, including di-(2-ethylhexyl) phthalate (DEHP) [4], di-*n*-octyl phthalate (DOP) [2] and DiNP [26], their complex metabolism to yield numerous products, predominantly oxidative metabolites, might explain the lower frequency and magnitude of detection in urine of their respective hydrolytic monoesters [17–21] compared with the monoesters of short-alkyl chain phthalates (e.g., diethyl phthalate (DEP), dibutyl phthalate (DBP)). Until specific biomarkers of exposure to isomeric phthalates are available, indirect measures of exposure to these phthalates may be valuable.

Quantification of the phthalic acid (PA) produced by hydrolysis of urinary phthalate metabolites has been used as an indirect indicator of exposure to phthalates [27]. The procedure involved a basic hydrolysis of the urine sample (10 mL) with sodium hydroxide (NaOH), acidification of the urine with hydrochloric acid (HCl), liquid–liquid extraction of the PA into diethyl ether, esterification of PA, and quantification of the derivatized PA with gas chromatography. One limitation of using PA as an indirect indicator of phthalate exposure is the lack of specificity of PA as a biomarker for any given phthalate. Because phthalates vary greatly in their toxicological properties, PA concentrations cannot be used for risk assessment purposes to estimate the likelihood, magnitude, and uncertainty of health risks associated with environmental exposures to phthalates. However, PA concentrations may be useful for establishing the presence of phthalates in the body or as an indirect indicator to estimate the prevalence of total exposure to phthalates.

Here, we report a method for assessing total exposure to phthalates, including the isomeric mixtures of high molecular weight phthalates, based on acid hydrolysis of the urinary phthalate monoesters to PA. After hydrolysis, PA is quantitated using our automated solid-phase extraction (SPE) coupled to isotope-dilution high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method [28].

2. Experimental

2.1. Reagents

PA, $^{13}C_2$-PA, D_4-PA, ammonium acetate (>98%), HCl (37%) and NaOH (97%) were purchased from Sigma-Aldrich Laboratories Inc. (St. Louis, MO, USA). Monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monocyclohexyl phthalate (MCHP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MOP), mono-(3-methyl-5-dimethylhexyl) phthalate (iso-nonyl, MiNP), mono-(3-methyl-7-methyloctyl) phthalate (iso-decyl, MiDP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), their $^{13}C_4$-labeled internal standards, D_4-DBP, D_4-DEHP, and D_4-DOP were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Mono-(3-carboxypropyl) phthalate (MCPP) and $^{13}C_4$-MCPP were obtained from Los Alamos National Laboratory (Los Alamos, NM, USA). Mono-iso-butyl phthalate (MiBP) and D_4-MiBP were generous gifts from Professor Jürgen Angerer (University of Erlangen-Nuremberg, Germany). Acetonitrile and water (HPLC grade) were purchased from Tedia (Fairfield, OH, USA), phosphoric acid (85%) was purchased from Fisher Scientific (Pittsburgh, PA, USA), formic acid (98% min, GR) was purchased from EM Science (Gibbstown, NJ, USA), ethyl acetate (99.8%) was purchased from Caledon (Ontario, Canada), and monosodium phosphate monohydrate (ultrapure bioreagent) and acetic acid (glacial) were purchased from J.T. Baker (Phillipsburg, NJ, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). Esterases from *Thermoanaerobium brockii*, *Mucor miehei*, and *Saccharomyces cerevisiae* were purchased from Fluka (St. Gallen, Switzerland).

2.2. Standards and sample preparation

Reagent solutions were prepared in acetonitrile and water using standard laboratory procedures. Stock solutions of D_4-PA and $^{13}C_2$-PA were prepared in acetonitrile and stored at −20 °C in Teflon-capped amber glass bottles until use. The $^{13}C_2$-PA internal standard spike solution (500 ng/mL) and the working standards were prepared in 1:9 acetonitrile:water from serial dilutions of the stock solutions and stored at 4 °C in Teflon-capped glass vials until use. Ten working standard solutions containing D_4-PA were prepared to encompass the entire linear range of the method (1 ng/mL to 1200 ng/mL). We used the peak area ratio of D_4-PA to $^{13}C_2$-PA (i.e., response factor (RF)) for quantification of PA. We used D_4-PA instead of native PA to eliminate the contribution from PA that may be introduced in the calibration standards during analysis. Calibration curves, prepared directly from the working standard solutions, of RF versus the reciprocal of the standard concentration ($1/x$) were used for quantification. The calculated PA concentrations were decreased by 2 ng/mL to correct for the PA present in the reagent blank after hydrolysis.

Urine (1 mL), spiked with $^{13}C_2$-PA internal standard solution (100 μL) and β-glucuronidase (5 μL in 250 μL of 1 M ammonium acetate buffer pH 6.5), was incubated at 37 °C for 90 min to allow for the deconjugation of the phthalate metabolite glucuronides. Reagent blanks were prepared and processed using the same procedure, except that urine was replaced with water.

2.3. Hydrolysis of phthalate monoesters using HCl

The deconjugated urine was treated with 400 μL of 37% HCl (equivalent to 4 mmol) and allowed to react for 180 min

at 90 ± 5 °C. After cooling to room temperature, the hydrolyzed urine sample underwent automated SPE as indicated below.

2.4. Hydrolysis of phthalate monoesters using NaOH

The deconjugated urine was treated with 250 μL of 5 M NaOH and allowed to react for 90 min at 90 ± 5 °C. Then, 500 μL of HCl was added to adjust the pH to ca. 2.0. After cooling to room temperature, the hydrolyzed urine sample underwent automated SPE as indicated below.

2.5. Hydrolysis of MEP using esterases

Three esterase solutions were made by dissolving in 100 mL of 0.01 M phosphate buffer (pH 8.0) 1 ng of *T. brockii* (2 U/g), 1 mg of *M. miehei* (1 U/mg), or 1 ng of *S. cerevisiae* (2 U/g). One milliliter of urine was spiked with 100 μL of $^{13}C_4$-MEP (5000 ng/mL) and 100 μL of $^{13}C_2$-PA internal standard solution. The spiked urine sample was combined with 250 μL of the enzyme solution (e.g., *T. brockii*) and allowed to react for 6 h at 37 °C. Then, 50 μL of 0.1 M phosphoric acid was added before automated SPE. The procedure was repeated with the other two esterases.

2.6. Automated SPE–HPLC–MS/MS

SPE was conducted on the Zymark RapidTrace Station (Zymark Corporation, Hopkinton, MA, USA) as previously reported [28]. HPLC–MS/MS was performed on a Waters Alliance 2690 liquid chromatograph (Waters Corporation, Milford, MA, USA) coupled with a ThermoFinnigan TSQ 7000 triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization interface as described before [28].

3. Results and discussion

First, we tested the effectiveness of several acids for hydrolyzing phthalate metabolites. Specifically, 1 mL of urine containing 30 ng/mL to 300 ng/mL of a mixture of 13 phthalate metabolites (i.e., PA, MMP, MEP, MCPP, MBP, MiBP, MCHP, MEHP, MOP, MNP, MiDP, MEHHP, MEOHP) was treated with either HCl (37%), formic acid (98%), acetic acid glacial, or phosphoric acid (85%) at 85 °C for 12 h. The urine was spiked after hydrolysis with 100 μL of a solution containing their corresponding isotope-labeled internal standards or with 100 μL of a solution of $^{13}C_2$-PA to calculate the concentration of each phthalate metabolite remaining after hydrolysis or the concentration of PA formed during the hydrolysis, respectively. Formic acid, acetic acid, and phosphoric acid failed to hydrolyze the phthalate monoesters; however, hydrolysis of phthalate monoesters to PA occurred when the urine was treated with HCl (Fig. 1). Next, we determined the efficiency of the hydrolysis of phthalate monoesters using three amounts of HCl (i.e., 3, 4, and 5 mmol) and found that 4 mmol was optimal. Last, to optimize the incubation time, we monitored the hydrolysis of the phthalate monoesters to

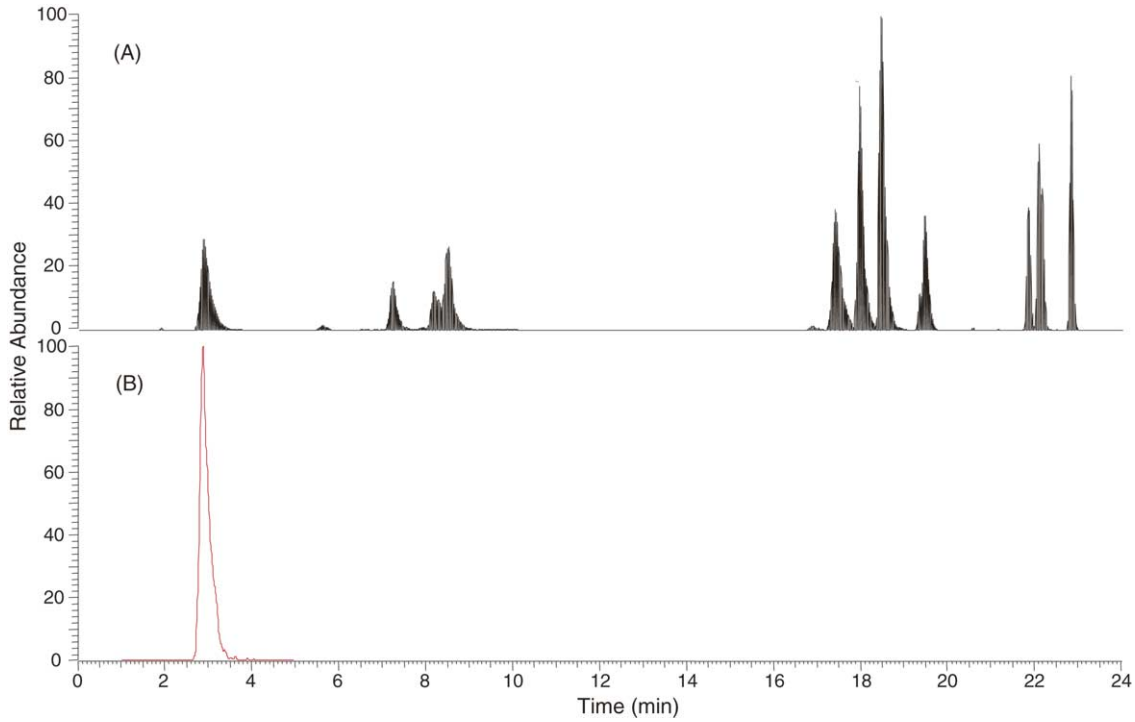

Fig. 1. Total ion chromatogram of a urine sample containing 30 ng/mL to 300 ng/mL of a mixture of 13 phthalate metabolites (i.e., PA, MMP, MEP, MCPP, MBP, MiBP, MCHP, MEHP, MOP, MNP, MiDP, MEHHP, MEOHP) before (A) and after (B) acid hydrolysis.

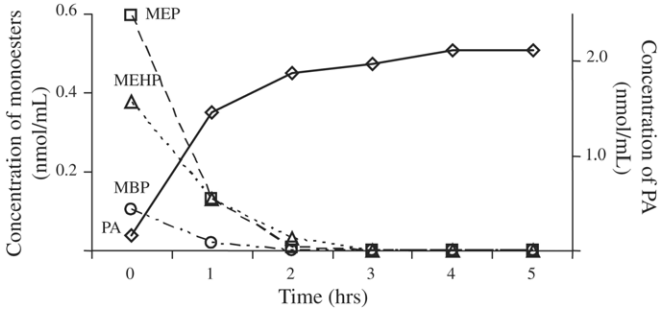

Fig. 2. Hydrolysis of select phthalate monoesters to phthalic acid (PA) using 4 mmol HCl.

PA using 4 mmol HCl for 5 h (Fig. 2). After 3 h, hydrolysis was complete for all of the phthalate metabolites tested. Furthermore, the percentages of hydrolysis, after 1 h and 2 h, respectively, were 89.5% and 99.5% (MMP), 77.9% and 97.8% (MEP), 79.8% and 84.9% (MCPP), 87.1% and 97.5% (MBP), 78.7% and 100% (MiBP), 39.8% and 54.9% (MCHP), 57.3% and 70.0% (MEHHP), 53.0% and 75.8% (MEOHP), 65.3% and 91.0% (MEHP), 64.0% and 74.5% (MOP), 55.0% and 75.5% (MiNP), and 50.0% and 71.3% (MiDP). Although the relatively small MMP and MEP hydrolyzed almost completely within 2 h, the larger MOP, MiNP and MiDP hydrolyzed only about 75% within the same time period. These data suggest that the rate of hydrolysis decreased with the increased length of the alkyl side chain.

We also evaluated basic and enzymatic hydrolysis of phthalate monoesters (using MEP as an example) and compared the efficacy of these hydrolyses with the one with HCl. To minimize the contribution from potential contamination, we used $^{13}C_4$-MEP (because $^{13}C_4$-PA could be produced only by hydrolysis of the spiked $^{13}C_4$-MEP). We estimated the efficacy of the acid and basic hydrolysis reactions at three $^{13}C_4$-MEP concentrations (500 ng/mL, 250 ng/mL, and 50 ng/mL); the enzymatic hydrolysis was evaluated only at 500 ng/mL. The hydrolysis efficacy was calculated from the ratio of the molar concentrations of $^{13}C_4$-PA and $^{13}C_4$-MEP. HCl and NaOH hydrolyzed most $^{13}C_4$-MEP to $^{13}C_4$-PA (Table 1). The conversion was quantitative for both HCl (>96%) and NaOH (>94%). In contrast, little $^{13}C_4$-MEP was hydrolyzed by the esterases (∼3%), suggesting that the enzymes were not suitable for the hydrolysis of phthalate metabolites.

Table 1
Comparison between the effectiveness of the acid, basic, and enzymatic hydrolysis of $^{13}C_4$-MEP

Additive	Concentration (ng/mL)		
	500	250	50
HCl	96.4 (1.2)	96.9 (3.3)	100.2(3.3)
NaOH	96.8 (2.1)	97.1 (2.6)	94.2(1.3)
Thermoanaerobium brockii	3.3 (2.8)		
Mucor miehei	2.8 (1.3)		
Saccharomyces cerevisiae	3.0 (1.8)		

Efficacy was calculated from the ratio of the molar concentrations of $^{13}C_4$-PA and $^{13}C_4$-MEP ($N = 3$) and expressed as a percentage (%). %R.S.D. in parenthesis.

Phthalates are ubiquitous environmental contaminants and can be incorporated into the urine during the collection, storage, and analysis processes. These diesters may be converted into PA during the hydrolysis reaction. Therefore, determining the contribution of diester contaminants in the total phthalate burden was important. We used D_4-phthalate diesters to determine the effect of the hydrolysis on the phthalate diesters by measuring the concentration of D_4-phthalate monoesters in the hydrolyzed urine sample. D_4-Phthalate monoesters could be produced only by hydrolysis of the spiked D_4-phthalate diesters. We monitored the hydrolysis reaction of D_4-DBP, D_4-DEHP and D_4-DOP with HCl and with NaOH (Table 2). The NaOH treatment resulted in a higher percentage of diester conversion to PA than the HCl treatment (Table 2). Therefore, to minimize the potential contribution from the phthalate diester contaminants, we performed the hydrolysis with HCl. Furthermore, the conversion of the relatively smaller phthalates (e.g., DBP) to PA was more efficient than that of the larger diesters (e.g., DEHP), similar to what we observed with the phthalate monoesters (vide supra). The amount of PA formed from DEHP during the HCl hydrolysis was only ∼3%. Therefore, even for contaminant DEHP, a major component in vinyl chloride plastic materials that may be present in laboratory supplies and equipment, its contribution to the total PA would not be significant. DBP may be found as a trace contaminant of solvents. Thus, solvent usage potentially could contribute to background contamination of DBP, which in turn, was hydrolyzed considerably to PA even with HCl (∼50%). However, the overall phthalate contribution of solvents and reagents, including both phthalate diesters and monoesters, estimated from the levels of PA (∼2 ng/mL) in the reagent blanks after the HCl hydrolysis, was relatively small and was subtracted from the concentrations of PA in the unknown samples.

The accuracy and precision of the measurements were established by determining the recovery of urine samples spiked with D_4-PA and by calculating the %R.S.D. of the repeated measurements, respectively (Table 3). The accuracy, obtained from triplicate measurements at two concentrations (50 ng/mL and 250 ng/mL), was calculated as $[PA]_b/[PA]_a$, where $[PA]_b$ and $[PA]_a$ are the concentrations of D_4-PA obtained from spiking the sample with the internal standard $^{13}C_2$-PA before and after hydrolysis, respectively. The accuracy was excellent at the two concentrations both for NaOH (95.8%–99.0%) and HCl (92.5%–98.7%). The inter-day precision for the acid hydrolysis ranged from 7.1% to

Table 2
Percentage of conversion of D_4-diesters (1000 ng/mL) to D_4-PA ($N = 3$)

Additive	D_4-DBP	D_4-DEHP	D_4-DOP
HCl	51.1 (3.7)	3.3 (2.9)	20.7 (6.4)
NaOH	72.3 (4.9)	42.6 (4.4)	56.3 (8.3)

%R.S.D. in parenthesis.

Table 3
Accuracy and precision of the D_4-PA measurements

Additive	Spiked concentration (ng/mL)	
	250	50
HCl[a]	98.7 (11.3)	92.5 (7.1)
NaOH[b]	99.0 (3.3)	95.8 (4.6)

[a] Interday precision, estimated as the %R.S.D. (in parenthesis) of triplicate measurements over 3 days.
[b] Intraday precision, estimated as the %R.S.D. (in parenthesis) of triplicate measurements.

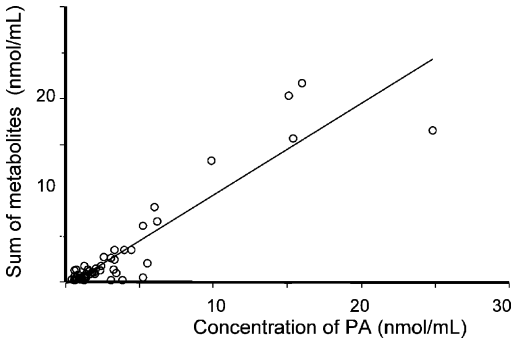

Fig. 3. Correlation analysis between the cumulative concentration of 13 phthalate metabolites (PA, MMP, MEP, MBP, MiBP, MCHP, MCPP, MEOHP, MEHHP, MEHP, MOP, MNP, MDP) before acid hydrolysis and the concentration of phthalic after hydrolysis. The correlation coefficient of the linear regression is 0.845.

11.3%, thus indicating the good reproducibility of the method (Table 3).

We analyzed 60 urine samples collected from anonymous volunteer adults with no known exposure to phthalates. First, using our method described previously [28], we determined the concentration of 13 phthalate metabolites (PA, MMP, MEP, MBP, MiBP, MCHP, MCPP, MEOHP, MEHHP, MEHP, MOP, MNP, MDP). Second, we estimated the concentration of PA after HCl hydrolysis. The correlation between the concentration of PA and the sum of concentrations of phthalate metabolites was good (correlation coefficient = 0.845) (Fig. 3). Furthermore, as expected, the concentrations of PA were higher than the sum of metabolites concentrations in most of the samples, thus confirming that some unidentified phthalate metabolites are present in urine. These results suggest that the determination of PA after hydrolysis of the urinary phthalate metabolites represents an adequate approach for assessing the total phthalate body burden.

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