Quantitative Detection of Nine Phthalate Metabolites in Human Serum Using Reversed-Phase High-Performance Liquid Chromatography–Electrospray Ionization-Tandem Mass Spectrometry

Kayoko Kato1, Manori J. Silva1,*, John W. Brock1, John A. Reidy1, Nicole A. Malek1, Carolyn C. Hodge1, Hiroyuki Nakazawa2, Larry L. Needham1, and Dana B. Barr1

1Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341 and 2Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Tokyo, Japan

Abstract

We developed a highly sensitive method for the quantitative detection of nine phthalate ester metabolites in human serum. This method requires denaturation of the serum enzymes immediately after blood collection to avoid the hydrolysis of the contaminant diester parent compounds introduced during blood collection and storage. Before analysis, the samples were subjected to an enzymatic deconjugation to hydrolyze the glucuronidated phthalate monoesters and a solid-phase extraction to isolate the monoesters from other serum components. The extracts were analyzed using reversed-phase high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry. The limits of detection of all nine phthalate monoesters in serum were in the low nanogram-per-milliliter range (0.6–1.3 ng/mL). Stable isotope-labeled internal standards for all analytes were used to improve precision and for recovery corrections. This highly selective method permits the analysis of phthalate monoesters without interferences resulting from the hydrolysis of the ubiquitous contaminant phthalate diesters by serum enzymes. In addition, it allows the direct measurement of the active phthalate monoester metabolites reportedly responsible for the reproductive and developmental toxicity of certain phthalates.

Introduction

The diesters of phthalic acid (phthalates) are industrial chemicals widely used in a variety of plastic products, paints, glue, hair sprays, nail polish, rocket fuel, insect repellants, cosmetics, perfumes, food packaging, and medical products (e.g., endotracheal tubes, ventilator tubing, and bags for intravenous solutions). Several phthalates and phthalate metabolites possess weak carcinogenic (1), teratogenic (2,3), antiandrogenic (4,5), and toxic (6–8) properties in animals. Because of the frequent use of phthalate-containing products, the majority of the U.S. population is potentially exposed to phthalates on a regular basis through diet, use of personal care products, and the environment (9). Phthalates are rapidly hydrolyzed in exposed humans and animals to their respective phthalate monoesters, which are further metabolized to other oxidative products, glucuronidated to increase the solubility, and excreted in urine or feces (10,11).

In order to evaluate the potential adverse effects of exposure to phthalates, accurate methods to measure the amount of the phthalates absorbed by the body must be developed. Exposure assessment using the diesters is difficult because of their extensive use and their ubiquitous presence in the environment. The diester contamination of serum can occur during sample collection and storage, as well as in the analytical stage from solvents and the environment, thus artificially increasing the measured concentrations. In contrast, measuring the phthalate monoester metabolites eliminates most of the contamination problems. Urinary monoester phthalate metabolites have been previously used for exposure assessment (12–16). However, because urine is a water intake-dependent unregulated fluid, toxicokinetics are often difficult to interpret from urinary data. Measurements in blood or blood products (i.e., serum and plasma) usually provide more easily interpretable data. However, blood or serum measurements of phthalate monoesters are susceptible to contamination from phthalate diesters that hydrolyze to their respective monoesters by serum enzymes. If the contamination is not properly eliminated, the serum monoester measurements will be artificially high.

We report a novel method for the accurate, precise, and sen-
sitive measurement of nine phthalate monoesters in human serum. This method involves the initial acid denaturation of the serum enzymes to eliminate esterase activity, avoiding hydrolysis of the diester phthalates and subsequently the production of artificially high concentrations of monoester phthalates from the hydrolysis of the phthalate diester contaminants introduced during the preservation, pretreatment, or analysis of the samples. Phthalate monoesters are extracted from the serum hydrolyzate using a mixed-phase solid-phase extraction (SPE) then concentrated and analyzed using high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS–MS).

Experimental

Materials

Native monoesters (> 98% purity), specifically monomethyl (MMP), monobutyl (MBP), monocyclohexyl (MCHP), monobenzyl (MBzP), mono-2-ethylhexyl (MEHP), monooctyl (MOP), mono-3-methyl-5-dimethylhexyl (isonylon, MNP), and mono-3-methyl-7-methyloctyl phthalate (isodecyl, MDP), and their 13C4-labeled internal standard analogues were purchased from Cambridge Isotope Laboratories (Andover, MA). Phthalate diesters dimethyl (DMP), diethyl (DEP), dibutyl (DBP), dicyclohexyl (DCHP), benzylibutyl (BzBP), di-2-ethylhexyl (DEHP), dioctyl (DOP), di-3-methyl-5-dimethylhexyl (isonylon, DINP), and di-3-methyl-7-methyloctyl phthalate (isodecyll, DiDP); ammonium acetate; and 4-methylumbelliferone glucuronide (> 98%) were purchased from Sigma-Aldrich (St. Louis, MO). β-Glucuronidase (Escherichia coli K12) was purchased from Roche Biomedical (Mannheim, Germany). HPLC-grade water, acetonitrile, and ethyl acetate were purchased from Fisher Chemicals (Pittsburgh, PA). Phosphoric acid, formic acid, and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, NJ).

Analytical standards and reagents

The procedure for the preparation of the standard solutions is explained elsewhere (14). The phthalate monoester stock solutions were stored at −20°C in Teflon-capped glass bottles until use. Ten unique standard solutions of native phthalate monoesters bracketing the desired analytical range for each analyte (0.1–2500 ng/mL), without exceeding the linear dynamic range of the method, were prepared in water from the stock solutions. The standard solutions were also stored at −20°C in a Teflon-capped glass bottle; the working solution was stored at 4°C. Stock and working solutions of the 13C4-labeled internal standards (monomethyl-13C4, monobutyl-13C4, monocyclohexyl-13C4, monobenzyl-13C4, monooctyl-13C4, mono-2-ethylhexyl-13C4, monocyclohexyl-13C4, mono-3-methyl-5-dimethylhexyl-13C4, and mono-3-methyl-7-methyloctyl-13C4 phthalate) were prepared similarly to the native standards and stored sealed at −20°C until use. The working internal standard solution was prepared in water as described for native standard solutions. This deconjugation internal standard was added to all samples to monitor β-glucuronidase enzyme activity as assessed by the levels of 4-methylumbelliferone released from the conjugated standard. β-Glucuronidase enzyme was used to hydrolyze glucuronidated metabolites. The deglucuronidation step was eliminated to measure free unconjugated phthalates. All standard solutions were prepared in glassware prerinsed with methanol.

The ammonium acetate buffer was prepared by dissolving ammonium acetate (38.6 g) in HPLC-grade water (200 mL) and adjusting to pH 6.5 with glacial acetic acid. Acidic buffer 1 (pH 2.0, 0.67M) was prepared by dissolving sodium phosphate-dibasic (40 g) and phosphoric acid (20 mL, 85%) in HPLC-grade water (1 L). Acidic buffer 2 (pH 2.0, 0.33M) was prepared by dissolving sodium phosphate-dibasic (20 g) and phosphoric acid (10 mL, 85%) in HPLC-grade water (1 L). Phosphoric acid (200mM) was prepared by diluting 2.07 mL of phosphoric acid to 100 mL with HPLC-grade water.

Methods

Human serum (1.00 mL) was thawed, acid treated to denature serum proteins (125 mL of 1M phosphoric acid), vortex mixed, sonicated (10 min), and spiked with isotopically labeled internal standards (100 mL, 0.24−1.0 ng/mL) and 4-methylumbelliferone glucuronide. The pH of the sample was subsequently optimized for β-glucuronidase enzyme activity with ammonium acetate buffer (3 mL). β-Glucuronidase enzyme (5 mL in 250 mL ammonium acetate buffer) was added, and the sample was incubated at 37°C for 90 min for deglucuronidation of the phthalate monooesters to occur. After enzymatic deconjugation, the samples were acidified with acidic buffer 1 (0.67M) and loaded onto an ABS ELUT-Nexus SPE cartridge (12 mL/200 mg, Varian, Harbor City, CA) and extracted using a vacuum manifold equipped with single-use Teflon flow lines (Supelco, Bellefonte, PA) (14). The phthalate monoesters were eluted with acetonitrile (2 mL), followed by ethyl acetate (2 mL), and then combined eluent was evaporated to dryness using a Turbovap (Zymark, Hopkinton, MA). After the drying step, the residue was resuspended in water (200 mL) and transferred to a glass autosampler vial for analysis. Phthalate-free microvial tips (Rainin, Oakland, CA) were used to aliquot samples and to add internal standard.

Instrument analysis

An aliquot of each sample (25 mL) was injected into a HPLC (Agilent 1100 series, Agilent Technologies, Palo Alto, CA) coupled with an API 3000 triple-quadrupole MS (PE Biosystems, Foster City, CA) for analysis by reversed-phase HPLC–ESI-MS–MS. The Sci-Ex API 3000 was operated in the TurboIonSpray mode with ionspray voltage at −4000 V, turbroprobe temperature at 400°C, and gas flow at 7 L/min.

The nine phthalate monoesters were chromatographically resolved using a Betasil phenyl HPLC column (5 μm, 50 mm × 2 mm, Keystone Scientific, Bellefonte, PA), with a nonlinear gradient program (17) from 100% aqueous (0.1% acetic acid in water) to 100% organic mobile phase (acetonitrile) at 0.6 mL/min. At the end of each run, the organic mobile phase was held at 100% for 0.5 min followed by equilibration with the
aqueous mobile phase for 1.5 min at 0.8 mL/min. In-line filters (2 and 5 μm, Upchurch Scientific, Oak Harbor, WA) were used to prevent particulate matter in the injected samples from entering the column. The 4-methylumbelliferone was analyzed to confirm the completion of the deglucuronidation process by β-glucuronidase enzyme.

Data processing, analysis, and quality control

The analyte peak was identified by matching the retention time with that of its respective 13C4-labeled internal standard. All data were automatically processed using the Analyst® software and manually evaluated. A calibration curve of peak-area ratio of analyte to stable isotope-labeled internal standard (after compensating for isotopic impurity of stable isotope-labeled internal standard) versus concentration ratio was used for quantitation. The chemical formula, retention time, and the analytical masses of the precursor and product ion combinations are listed in Table I.

Quality control (QC) materials were made using pooled acid denatured bovine serum spiked with monoester analytes. Two QC pools (QC-low and QC-high) with all nine analytes were made and transferred into 5-mL glass vials, which were capped and sealed for future use. Both QC-low and QC-high materials were repeatedly extracted and analyzed (N > 50) to generate QC-lows and sealed for future use. Both QC-low and QC-high materials were re-extracted.

Method validation

Recovery calculation. Recovery was calculated by using the ratio of the amount of analytes recovered after SPE to the amounts originally added. The internal standard mixture containing 13C4-labeled analytes were added before SPE to obtain the original amount and after the drying step to obtain the recovered amount.

Evaluation of the necessity of 60% formic acid to release conjugated phthalates from serum proteins. Aliquots of serum (1 mL) were spiked with a mixture of phthalate monoesters containing MMP, MEP, MBP, MBzP, MCHP, MOP, MNP, and MDP and equilibrated 48 h at 37°C. Different amounts (0, 10, 20, 50, 75, and 100 μL) of 60% formic acid were added in triplicate to serum aliquots to release conjugated phthalates, and the samples were analyzed for the above phthalate monoesters.

Evaluation of serum enzyme activity on diester hydrolysis. Aliquots of serum (1 mL) were spiked with a mixture of phthalate diesters containing DMP, DEP, DBP, DEHP, DCHP, DBzP, DOP, DiNP, and DiDP (5000 ng/mL), and monoesters were analyzed as described at different time intervals after spiking. The analyses were repeated with diester spiked serum samples excluding the incubation step and also after replacing serum with water.

The procedure was repeated with spiked serum samples from three individuals with BzBP (5000 ng/mL). The samples were kept at ~4°C for 24 h, incubated for 90 min at 37°C, and extracted as previously described. The extracts were then analyzed for MBP and MBzP.

Evaluation of gamma ray irradiation. To study the effectiveness of gamma ray irradiation on deactivating the serum enzymes, the human serum was dispensed into borosilicate glass test tubes and irradiated with gamma irradiation (1 × 106 RADS) for 50 min using a Gammacell 220, AECL industrial irradiator (MDS Nordion Inc., Toronto, ON, Canada). Aliquots (1 mL) of these irradiated samples were spiked with a mixture of phthalate diesters containing DMP, DEP, DBP, DEHP, DCHP, DBzP, DOP, DiNP, and DiDP (5000 ng/mL) and analyzed at different time intervals. All analyses were done in triplicate.

Evaluation of phosphoric acid as the serum-denaturing agent. To determine the amount of phosphoric acid required to completely deactivate the serum enzymes, different amounts of phosphoric acid (0–200 μmol) were added to 1-mL aliquots of human serum. The samples were then spiked with a mixture of phthalate diesters containing DMP, DEP, DBP, DEHP, DCHP, DBzP, DOP, DiNP, and DiDP (5000 ng/mL) and analyzed after 24 h of spiking.

To analyze the effect of phosphoric acid on the hydrolysis of monoester metabolites, the acid-treated human serum aliquots (1 mL) were spiked with a mixture of phthalate monoesters containing MMP, MEP, MBP, MCHP, MBzP, MCHP, MOP, MNP, and MDP and analyzed after 24 h. The internal standard was added before and after acid treatment for total phthalate quantitation and residual phthalate quantitation, respectively.

Results and Discussion

We developed a sensitive method to assess human exposure to phthalates by analyzing their monoester metabolites in
serum. Unlike urine, serum samples have active esterases that can hydrolyze both phthalate diesters into their respective monoesters, and phthalate monoesters to phthalic acid. The highest esterase activity in serum was observed at normal serum pH (pH ~ 8.0).

We detected a rapid increase in monoester levels after spiking diesters into untreated human serum (Figure 1). The rate of increase in monoester levels was more pronounced for the shorter alkyl chain phthalates such as DMP, DEP, and DBP. We observed a hydrolysis during the 90-min incubation period at 37°C for DBP (> 80%), DMP (> 60%), and DEP (> 20%). The diester hydrolysis was less than 5% for DEHP, DNP, and DDP. Interestingly, we observed a lower rate of hydrolysis when we omitted the 90-min incubation at 37°C (Figure 2). In a separate experiment, spiking the serum with BzBP resulted in an uneven breakdown of the diester producing 90% MBzP and 10% MBP (Table II).

Analysis of buffered water spiked with diesters did not generate monoesters after incubation with β-glucuronidase, indicating the diesters are hydrolyzed by components endogenous in the serum, presumably serum esterases, and not by the β-glucuronidase enzyme added to deglucuronidate the monoesters. We initially attempted to deactivate the serum esterases by using gamma irradiation at 1 × 10⁶ RADS for 50 min. Although irradiation of diester-spiked serum samples re-

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**Table II. The Resultant Serum MBP and MBzP Levels Generated by Hydrolysis of BzBP by Serum Enzymes upon Spiking Serum Samples from Three Subjects with BzBP (5000 ng/mL)**

<table>
<thead>
<tr>
<th></th>
<th>Mean conc.</th>
<th>% CV</th>
<th>% generated</th>
<th>Mean conc.</th>
<th>% CV</th>
<th>% generated</th>
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</thead>
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<tr>
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<td>2760</td>
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<td>208.3</td>
<td>1.6</td>
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<tr>
<td>Subject 2</td>
<td>2903</td>
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<td>93.31</td>
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<td>90.51</td>
<td>325.0</td>
<td>1.2</td>
<td>9.49</td>
</tr>
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**Figure 1.** Concentrations of phthalate monoesters at different time intervals after spiking with 5000 ng/mL mixture of DMP, DEP, DBP, DCHP, DOP, DNP, and DDP. Concentration at time 0 indicates the initial concentration of monoesters in the absence of diester spiking. Error bars represent standard deviation of triplicate extractions.

**Figure 2.** The concentration of MMP showing an increase in serum lipase activity during incubation at 37°C for 90 min. Repeat experiment with water replacing serum, excluded participation of β-glucuronidase in the diester hydrolysis process. Similar results were found for other analytes. Error bars represent standard deviation of triplicate extractions.

**Figure 3.** Concentration of phthalate monoesters in diester-spiked serum samples upon addition of different µmoles of H₃PO₄ to de-activate the serum enzymes. The minimum amount of 100 µmoles of H₃PO₄ was needed to complete the de-activation process. Error bars represent standard deviation of triplicate extractions.

**Figure 4.** A Shewart plot of quality control for MMP in human serum. The QC was characterized with multiple separate analyses and subsequently used to monitor long-term accuracy and precision of the assay.
suited in a decrease in the rate of diester hydrolysis, the enzyme denaturing process was incomplete. We observed continued diester hydrolysis producing monoester phthalates from diester-spiked serum samples. Hence, we abandoned our attempts to deactivate serum esterases by gamma irradiation.

We successfully deactivated the serum enzymes by treating the serum with phosphoric acid (1 mL serum/125 μmol H₃PO₄) followed by neutralization before deglucuronidation. Furthermore, we optimized the concentration of phosphoric acid required to denature serum lipases without further hydrolyzing the monoester analytes by studying the effect of acid strength on the enzyme hydrolysis process (Figure 3). At high concentrations, phosphoric acid could facilitate the hydrolysis of both diesters and monoesters. Monoester and diester spike studies in the presence and absence of serum confirmed that there was no apparent acid hydrolysis of monoesters or contaminant diester phthalates upon addition of 125 μmol of phosphoric acid to 1 mL of serum. In addition, the analytes may bind to serum proteins, making the active sites unavailable for sorbent interaction during SPE. The acid treatment during the extraction process released monoesters equally effectively as 60% formic acid. Hence, we eliminated the formic acid treatment from the method.

We evaluated the serum matrix effect on the calibration curve by analyzing the standards prepared both in water and human serum. Standards spiked in serum produced calibration curves with slopes not significantly different from those produced by standards prepared in water. Hence, the calibration curves were produced by directly analyzing standards prepared in water.

The SPE recoveries for most phthalate monoesters from human serum were excellent (Table III). We observed a low recovery for MMP, probably because of its hydrophilicity compared with the other monoesters. Isotope dilution with ¹³C₄-labeled isotopes corrected for the recovery variations from differences in the composition of serum samples. We calculated the method limit of detection (LOD) as 3σ₀, where σ₀ is the standard deviation when the concentration approaches zero (19). The LODs for analysis of phthalate monoesters in serum ranged from 0.6 to 1.3 ng/mL (Table II) and were comparable with those obtained in urine (14).

Acid-treated QC materials remained stable for at least three months. The monoester concentration associated with diester or monoester hydrolysis did not appear to increase or decrease. The QC plot for MMP in the low QC pool is shown in Figure 4. This plot reflects both intra- and interday variations over a period of three weeks.

Conclusions

We used our newly developed method to analyze eight monoester phthalates in a selected human population and found detectable concentrations of MEP, MBP, and MEHP in most serum samples tested (Figure 5). We observed higher phthalate monoester levels in non-acid treated frozen serum samples spiked with diesters compared with acid treated frozen serum samples. The hydrolysis may have occurred during the thawing period. Therefore, serum samples for phthalate analysis should be acid-treated immediately after collection and before freezing. This sensitive method for the detection of phthalates in serum will enhance the exposure studies to determine relevant health effects.

Table III. Recoveries and LOD of Monoester Phthalates

<table>
<thead>
<tr>
<th>Spike concentration (ng/mL)</th>
<th>40</th>
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<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
<td>Recovery (%)</td>
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<tr>
<td>MMP</td>
<td>64.2</td>
<td>13.2</td>
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<td>MEP</td>
<td>93.2</td>
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<td>MBP</td>
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<td>MBzP</td>
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Acknowledgment

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References


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