Article

Development and Validation of an Analytical Method for Quantitation of Monobutylphthalate, a Metabolite of Di-*n*-Butylphthalate, in Rat Plasma, Amniotic Fluid, Fetuses and Pups by UPLC–MS–MS

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Abstract

Phthalates have been used for decades as softening agents in the production of plastics, but in recent years have been extensively investigated for their potential hazards to human health and the environment. Di-*n*-butyl phthalate (DBP), with widespread exposure occurring through a variety of consumer products such as cosmetics and pesticides, is a suspected carcinogen and an endocrine system disruptor in both humans and laboratory animals. Its predominant metabolite is the monoester, monobutyl phthalate (MBP), which can serve as a marker of exposure. To support toxicological studies of DBP in pregnant and lactating rats and their offspring, a novel ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS–MS) method was developed and validated for quantitation of MBP in rat plasma, amniotic fluid, fetuses and whole pup samples. Plasma samples were extracted using a simple protein precipitation with acetonitrile. Extraction and delipidation of pup homogenate was performed using acetonitrile and then submerging the vials in liquid nitrogen. Extracts were analyzed by UPLC–MS–MS in the negative ion mode. The method was successfully validated over the concentration ranges 25–5,000 ng/mL in female Sprague Dawley (SD) rat plasma and 50–5,000 ng/g in SD pup homogenate. Matrix calibration curves were linear ($r \geq 0.99$), and the percent relative error (%RE) values were $\leq \pm 15\%$ for standards at all levels. Absolute recoveries were $> 92\%$ in both matrices. The limits of detection (LODs) were 6.9 ng/mL in plasma and 9.4 ng/g in pup homogenate. Acceptable intra- and interday accuracy and precision were demonstrated by mean %RE $\leq \pm 7.5$ and relative standard deviation (%RSD) $\leq 10.1\%$. Extract stability was demonstrated for ~ 6 days at various temperatures and freeze–thaw stability was demonstrated after 3 cycles over 3 days. Secondary matrix evaluation was performed for MBP in amniotic fluid and pooled fetus homogenate (mean %RE $\leq \pm 11.5$ and %RSD ≤ 13.7). These data demonstrate that this simple method is suitable for determination of MBP in plasma, amniotic fluid, fetus and pup samples from toxicological studies of DBP.

Introduction

Phthalates have been used for decades as softening agents in the production of plastics. It is estimated that over 470 million pounds are produced or imported each year in the USA (1). Di-*n*-butyl phthalate (DBP) is found in a variety of consumer products such as plastic food wrap, adhesives, raincoats, shower curtains, nail polish and other cosmetics and insect repellents. Because the phthalate molecules are not covalently bound to the plastic material, they can readily leach out into the environment. DBP levels in air over New York City have been detected at 3.3–5.7 ng/m³, and in some drinking water supplies at 0.1–5 ppb (2). Phthalate metabolites are often some of the most ubiquitous and abundant xenobiotics found in human urine; median urine concentrations are often in the 10–100 ng/mL range for individual metabolites (3–5). The predominant route of human exposure to DBP occurs through diet (from food packaging or through the food chain), but can also occur through dust ingestion, inhalation and dermal absorption (6–9). Typical human exposure is estimated at 2–10 µg DBP/kg bw/day, with higher exposures estimated in infants and children. In addition, exposures in women of child-bearing age were found to be higher than other age or sex groups (10) and fetal exposure during pregnancy is suspected (11–14).

DBP is a suspected carcinogen and an endocrine system disruptor in both humans and laboratory animals. Severe and permanent anti-androgenic effects on male reproductive development and function have been documented following prenatal exposure to DBP. For example, malformed external genitalia (hypospadias), undescended testis (cryptorchidism), spermatogenesis impairment and anorectal malformations have been found in some male rat offspring following prenatal exposure to DBP (15–17). However, more data are needed to better understand the reproductive and developmental effects that occur following DBP exposure.

DBP is rapidly metabolized *in vivo* to the monoester, monobutyl phthalate (MBP), which can serve as a marker of exposure and which has also been suggested to be responsible for its toxic effects (18–19). MBP is further metabolized to a glucuronide conjugate (MBP-G), but levels in maternal plasma, amniotic fluid, placenta and embryo were much lower than for MBP, and MBP-G is not considered biologically active (11, 13). While measurement of total MBP, following enzymatic hydrolysis of MBP-G, would give a more accurate determination of MBP in a biological sample, measurement of free MBP can serve as a simple marker of exposure and fetal and lactational transfer in toxicological studies. Methods for measuring MBP in biological samples such as urine and plasma have included gas chromatography–mass spectrometry (GC–MS) (20), liquid chromatography–mass spectrometry (LC–MS) (21) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (18–19, 22–23). However, sample volumes are often rather large (e.g., 100 µL) to achieve adequate sensitivity, or limits of quantitation are set very high (e.g., 260 ng/mL) such that phthalate background do not interfere with analysis. The objective of this work was to develop and validate an analytical method for sensitive and accurate quantitation of free MBP in rat plasma, amniotic fluid, fetuses and pups in support of toxicological studies of DBP in pregnant and lactating rats and their offspring.

Experimental

Chemicals and reagents

Monobutyl phthalate (MBP, >97% purity) was purchased from Sigma-Aldrich (Saint Louis, MO), and monobutyl phthalate-*d*₄

Figure 1. Structures of MBP and MBP-*d*₄ (IS).

(MBP-*d*₄, internal standard, IS) was from Toronto Research Chemicals (Ontario, Canada). The molecular structures of MBP and MBP-*d*₄ are shown in Figure 1. High-purity solvents were used to minimize the potential for phthalate contamination. Optima LC–MS grade acetic acid, acetonitrile and water were from Fisher Scientific (Pittsburgh, PA). Formic acid (98%) was from Fluka/Sigma-Aldrich (Saint Louis, MO). High-purity methanol and HPLC-grade isopropanol and acetone were from B&J/Honeywell (Morristown, NJ). K3 EDTA female Sprague Dawley (SD) rat plasma and amniotic fluid were purchased from Bioreclamation (Liverpool, NY). Naïve male and female rat pups (postnatal day 4) and gestation day 18 fetuses were purchased from Hilltop Lab Animals (Scottsdale, PA).

Analytical method validation

Experimental design

The method was validated in female rat plasma and pup homogenate by analyzing calibration standards and quality control (QC) samples in replicates over multiple analysis days to demonstrate linearity, selectivity, sensitivity, recovery, intra- and interday precision and accuracy and reproducibility over the desired concentration ranges. The concentration ranges were 25–5,000 ng/mL in plasma and 50–5,000 ng/g in pup homogenate. Selectivity was assessed by analyzing triplicate matrix blanks of different lots with and without IS for background interferences. The lower limit of quantitation (LLOQ) was the lowest standard that could be accurately quantitated within 20% of the nominal value and at which six replicates could be reproduced within 20% relative standard deviation (RSD). The limit of detection (LOD) was defined as three times the standard deviation of the LLOQ response expressed as concentration. Absolute recovery was expressed as the percentage of the response of the analyte prepared in matrix to the response of the analyte prepared in solvent at the same concentration levels. Intraday precision (%RSD) and accuracy (percent relative error, %RE) were evaluated at six concentration levels using three independent standards prepared on the same day. Interday precision and accuracy were evaluated at the same six concentration levels, using multiple sets of independent standards

prepared and analyzed over multiple analysis days. Instrument drift (reInjection reproducibility) was assessed by splitting one of the sets of calibration standards and running them at the beginning and end of the sample set, with multiple samples run in-between. The first set was used as the calibration standards, and the later 'split set' was analyzed as an independent QC set to assess drift. The method was also evaluated for secondary matrices, amniotic fluid and fetus homogenate. All samples were analyzed by UPLC-MS-MS as described below.

Preparation of stock solutions

To reduce the potential of exogenous phthalate contamination, solutions were prepared and stored in glass. Solvent bottles, flasks and graduated cylinders were first washed and rinsed thoroughly using an automated dishwasher. A working solution of the IS was prepared at 5 µg/mL in water. Standard stock solutions of MBP in 50:50 methanol:water were prepared at 500 µg/mL and 200 µg/mL. Spiking solutions were prepared in water from alternating MBP stock solutions and were used to prepare the matrix calibration standards and QC samples. The ranges for these spiking solutions were 0.025–5 µg/mL for plasma standards, or 1–1,000 µg/mL for pup homogenate standards.

Preparation of plasma calibration standards

Plasma calibration standards and QC samples were prepared by spiking 25 µL of blank plasma with 25 µL of the appropriate spiking standard to obtain plasma concentration levels of 25, 50, 100, 1,000, 2,500 and 5,000 ng/mL. Sample extraction was achieved by addition of 25 µL of the IS working solution to the 50 µL of diluted matrix, followed by 425 µL of 0.1% formic acid in acetonitrile, vortex mixing and centrifugation for 6 min; the supernatants were removed for analysis.

Secondary matrix evaluation in amniotic fluid

Triplicate matrix samples containing MBP were prepared at three concentrations in amniotic fluid (100, 1,000 and 2,500 ng/mL) and analyzed using a plasma calibration curve. The amniotic fluid samples were prepared the same way as the plasma samples described above, using 25 µL of blank amniotic fluid.

Preparation of pup calibration standards

Pup homogenate used for validation was prepared from at least two pups of each sex. Thawed tissue was cut into relatively uniform pieces prior to homogenization. Calibration standards and QC samples were prepared by spiking 0.5 g of pup homogenate with 25 µL of the appropriate spiking standard to obtain concentration levels of 50, 100, 500, 1,000, 2,500 and 5,000 ng/g. To each sample, 25 µL of the IS working solution and 300 µL of 0.1% formic acid in water were added. Each vial was vortexed briefly and then sonicated for 90 min. Sample extraction was achieved by addition of 1 mL of acetonitrile, vortex mixing and centrifugation for 15 min. Each supernatant was decanted, filtered through a 0.45 µm PVDF filter, equilibrated in liquid nitrogen to facilitate lipid separation and centrifuged again. The clarified supernatants were removed for analysis.

Secondary matrix evaluation in fetus homogenate

A matrix evaluation was performed for fetus homogenate using the validated pup homogenate method. Fetuses were pooled by litter and then homogenized. The fetal homogenates were prepared for analysis the same way as the pup homogenates described above, using 0.5 g of blank fetus homogenate. Triplicate fetal homogenates were prepared

at three concentrations (100, 500 and 2,500 ng/g) and analyzed using a pup homogenate calibration curve.

Dilution verification

Based on anticipated concentrations in study samples, a dilution verification was conducted at 516,000 ng/mL in plasma, 7,500 ng/g in pup homogenate and 25,200 ng/g in fetus homogenate. The samples were extracted as above, diluted either 1:200 with blank plasma extract, 1:2 with blank pup homogenate extract, or 1:10 with blank fetus homogenate extract and analyzed by UPLC-MS-MS.

Stability

Freeze-thaw stability was performed using sets of triplicate non-extracted matrix samples at three concentrations (50, 100 and 2,500 ng/mL in plasma; 100, 500 and 2,500 ng/g in pup homogenate) stored frozen at -20°C and subjected to three freeze-thaw cycles over 3 days. The determined concentrations were compared to that of a fresh (day 0) sample. Additional stability assessment was performed at two concentrations (31.0 and 3,750 ng/mL in plasma and 62.5 and 3,750 ng/g in pup homogenate) in triplicate and evaluated at predetermined intervals ~60 days. Extracted matrix samples were evaluated following ambient and refrigerated storage for 6 days, which represented the expected duration of study sample analysis.

LC-MS-MS analysis and analyte quantitation

The UPLC-MS-MS system consisted of a Waters (Milford, MA) Acquity UPLC coupled to a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray (electrospray) source (Sciex, Framingham, MA). Chromatographic analysis was performed using a Waters Acquity UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8-µm particle size) and a Waters Acquity UPLC HSS T3 guard column (5 mm × 2.1 mm i.d., 1.8 µm particle size). Five microliters of sample were injected onto the column and elution was achieved at ambient temperature using a binary gradient and a flow rate of 0.5 mL/min. The needle washes consisted of 60:30:10 acetonitrile:isopropanol:acetone and 0.1% formic acid in water. The mobile phases consisted of (A) water with 0.02% acetic acid and (B) acetonitrile with 0.02% acetic acid. The gradient was 30% B for 0.5 min, 30 to 95% B from 0.5 to 3 min, hold at 95% B for 1 min. The electrospray ion source was operated in negative ion mode with an ion spray voltage of -3,700 V and source temperature of 600°C. The curtain gas was 15 psi, the nebulizer gas was 60 psi, the auxiliary gas was 40 psi and the interface heater was on. The selected multiple reaction monitoring (MRM) ion transitions were 221 → 77 for MBP, and 225 → 81 for MBP-d₄ (IS). MRM transition for MBP-glucuronide (397 → 221) was also included in the method for non-quantitative, diagnostic purposes. The optimized compound-dependent parameters for MBP and MBP-d₄ were declustering potential = -35 V; entrance potential = -10 V; collision energy = -26 V; and collision cell exit potential = -3 V. The Analyst software version 1.4.2 (Sciex) was used for data acquisition and analysis.

Calibration curves were generated by plotting the peak area ratios of MBP to IS as a function of analyte concentration. The regression model for MBP was a linear weighted least squares algorithm with a weighting factor of 1/concentration-squared (1/x²). The concentration of MBP was calculated using response ratio, the regression equation, initial sample volume and dilution when applicable. Plasma and amniotic fluid data are expressed as ng/mL, while pup and fetus data are expressed as ng/g.

Figure 2. Representative UPLC–MS–MS chromatograms for MBP in plasma, (a) blank rat plasma, (b) blank rat plasma with IS, (c) plasma spiked at 25 ng/mL and (d) plasma spiked at 5,000 ng/mL. Insets are expanded views.

Results and Discussion

Method development

The method was initially adapted from Fennell et al. (23). However, different column chemistries and dimensions were evaluated for optimum peak shape and resolution from background interference. A Waters UPLC HSS T3 column was finally selected. The method for extraction and delipidation of pup and fetus homogenate was adapted from previous work in our laboratory (24), but different

solvents were used to maximize solubility and extraction efficiency and to match mobile phase conditions. Sample amounts were minimized; only 25 μ L of plasma or amniotic fluid, or 0.5 g of pup or fetus homogenate were required. The final method requires only a single-step protein precipitation for plasma and amniotic fluid, and simple extraction and delipidation of pup and fetus homogenate, yet improved sensitivities, reduced background interference and reduced sample volumes over existing methods was achieved.

Figure 3. Representative UPLC-MS-MS chromatograms for MBP in pups, (a) blank rat pup homogenate, (b) blank rat pup homogenate with IS, (c) pup homogenate spiked at 50 ng/g and (d) pup homogenate spiked at 5,000 ng/g. Insets are expanded views.

Method validation

Each of the blank matrices, as well as solvent blanks, showed detectable levels of MBP, indicating background interference, not unexpected given the ubiquity of phthalates in the environment. Throughout the study, sources of excessive background were identified in the acetic acid, water and plastic bags containing the fetuses and amniotic fluid. Presence of the IS (MBP- d_4) did not contribute to the MBP background. A selectivity threshold was set at 40%, whereby blank subtraction was required if MBP responses in the

blanks rose above 40% of the responses for the LLOQ standards. Representative chromatograms are presented in Figures 2 and 3. Method validation data are summarized in Table I. The LLOQ for MBP was 25.0 ng/mL for plasma and 50.0 ng/g for pup homogenate; the LODs were 6.9 ng/mL in plasma and 9.4 ng/g in pups.

The calibration curves in plasma and pup homogenate were linear with correlation coefficients $r > 0.99$. The intra- and interday precisions were $<10.1\%$ RSD in each matrix and accuracies were within $\pm 7.5\%$ RE. It should be noted that MBP background was high for the

Table I. Method Validation Data for MBP in Female Rat Plasma and Pup Homogenate

Validation parameter	Plasma	Pup homogenate
Matrix concentration range (ng/mL or ng/g)	25.0–5,000	50.0–5,000
LOD (ng/mL or ng/g) ^a	6.9	9.4
LLOQ (ng/mL or ng/g) ^b	25.0	50.0
Correlation coefficient (<i>r</i>)	≥0.99	≥0.99
Mean absolute recovery (%)	92.2	102
Precision and accuracy ^c		
Intraday Precision (%RSD) ^d	≤6.1	≤10.1
Intrasay Accuracy (Mean %RE) ^e	≤±7.0	≤±7.5
Interday Precision (%RSD)	≤7.9	≤7.6
Interday Accuracy (Mean %RE)	≤±7.0	≤±4.0
Dilution Verification ^f		
Precision (%RSD)	≤3.1	≤0.5
Accuracy (Mean %RE)	≤±8.7	≤±2.6
Secondary Matrix Evaluation (Amniotic Fluid or Fetus Homogenate) ^g		
Precision (%RSD)	≤8.2	≤13.7
Accuracy (Mean %RE)	≤±11.5	≤±3.4

^aLOD is limit of detection.^bLLOQ is lower limit of quantitation; lowest standard at which RE ≤ ±20% and RSD ≤ 20% for *n* = 6.^cPrecision and accuracy determined for triplicate QCs at six levels in each matrix: 25, 50, 100, 1,000, 2,500 and 5,000 ng/mL (plasma) and 50, 100, 500, 1,000, 2,500 and 5,000 ng/g (pup homogenate).^d%RSD is percent relative deviation.^e%RE is percent relative error.^fPrecision and accuracy determined for triplicate QCs at 516,000 ng/mL (plasma), 7,500 ng/g (pup homogenate) and 25,200 ng/g (fetus homogenate).^gPrecision and accuracy for secondary matrices determined for triplicate QCs at three levels in each matrix: 100, 1,000 and 2,500 ng/mL (amniotic fluid) and 100, 500 and 2,500 ng/g (fetus homogenate).**Table II.** Stability data for MBP in female rat plasma and pup homogenate

Stability endpoint	Mean % of day 0 (%RSD)	
	Plasma	Pup homogenate
Extract stability		
Refrigerated extracts (4°C, 6 days)	99.4–106 (%RSD ≤ 6.1%)	104–106 (%RSD ≤ 3.7%)
Ambient extracts (RT, 6 days)	105–107 (%RSD ≤ 6.1%)	102–103 (%RSD ≤ 4.3%)
Matrix stability		
Freeze-thaw (3 cycles over 3 days)	82.5–97.7 (%RSD ≤ 6.1%)	90.5–99.9 (%RSD ≤ 9.7%)
Frozen matrix (−20°C, 60 days)	98.5–102 (%RSD ≤ 7.5%)	92.8–104 (%RSD ≤ 9.0%)

Extract and freeze-thaw stability determined for triplicate QCs at three levels: 50, 100 and 2,500 ng/mL (plasma) and 100, 500 and 2,500 ng/g (pup homogenate). Frozen matrix stability determined for triplicate QCs at two levels: 31.0 and 3,750 ng/mL (plasma) and 62.5 and 3,750 ng/g (pup homogenate).

third batch of validation samples in plasma (responses in the blanks were >40% of the responses in the LLOQ standards), so background subtraction was performed for that batch. Similar linear regression equations and accuracies were achieved for all three batches, and interday precision was excellent (≤ 7.9% RSD), indicating that the presence of the phthalate background and need for subtraction did not appear to impact the results. Recovery was determined at all six calibration levels in each matrix. The mean absolute recoveries for MBP were 92.2% in plasma (7.1% RSD) and 109% (7.5% RSD) in pup homogenate. For both plasma and pup homogenate, a set of matrix standards was split before analysis to assess instrument drift. The relative difference between the determined concentrations for the split set compared to the calibration set were within ±2.5% for pup tissue and ± 7.8% (15.4% at the LLOQ) for plasma, indicating no instrument drift. Diluted plasma extracts prepared at 516,000 ng/mL gave an average back-calculated value of 561,000 ng/mL (8.7% RE, 3.1% RSD), demonstrating the ability to dilute samples into the range of the calibration curve. Likewise, pup samples prepared at 7,500 ng/g could be diluted into range with acceptable accuracy and

precision; average back-calculated values were 7,690 ng/g (2.6% RE, 0.5% RSD).

Matrix evaluation in amniotic fluid and fetus homogenate

Successful matrix evaluations were achieved for MBP in amniotic fluid and pooled fetuses. Amniotic fluid samples were prepared at 100, 1,000 and 2,500 ng/mL and quantitated against the plasma calibration curve; RE and RSD were ≤ ±11.5% and ≤ 8.2% RSD, respectively. Fetus samples were prepared at 100, 500 and 2,500 ng/g MBP and quantitated against the pup homogenate calibration curve; RE and RSD were ≤ ±3.4% and ≤ 13.7%, respectively. Both amniotic fluid and fetus control blanks showed elevated MBP background (responses in the blanks were > 40% of the responses in the corresponding plasma or pup LLOQ standards). Blank subtraction was thus performed for the amniotic fluid and fetus validation standards. Fetus samples prepared at 25,200 ng/g could be diluted into range with acceptable accuracy and precision; average back-calculated values were 28,600 ng/g (13.6% RE, 8.4 %RSD).

Stability

Studies were performed to evaluate both matrix and extract stability of MBP. The determined concentrations of the stability samples were compared to those of freshly prepared samples ('day 0'), and data are presented in Table II. Extracts were 99.4 to 107% of day 0, demonstrating that MBP is stable ~6 days at ambient and refrigerated temperatures. MBP in plasma and pup homogenate stored undergoing three freeze-thaw cycles over 3 days were within 82.5–99.9% of day 0. MBP in plasma and pup homogenate stored frozen (–20°C) for 60 days were within 92.8 to 104% of day 0 demonstrating the stability over these conditions and durations.

Conclusion

MBP, the predominant metabolite of di-*n*-butyl phthalate, can be precisely and accurately quantitated in rat plasma, amniotic fluid, pup and fetus homogenate using this validated UPLC–MS–MS method. The quantitation ranges are 25–5,000 ng/mL for plasma and amniotic fluid and 50–5,000 ng/g for pup and fetus homogenate. In addition, samples above the upper limit of quantitation can be accurately diluted into the calibration range for analysis. The method requires only a single-step protein precipitation for plasma and amniotic fluid and simple extraction and delipidation of pup and fetus homogenates. While measurement of total MBP, following enzymatic hydrolysis of MBP-G, would give a more accurate determination of MBP in the sample, measurement of free MBP with this method serves as a simple marker of exposure/transfer in the supported toxicological studies of DBP in pregnant and lactating rats and their offspring.

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