Validation and Application of an HPLC Method for Determination of Di (2-ethylhexyl) Phthalate and Mono (2-ethylhexyl) Phthalate in Liver Samples

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Abstract

A reversed-phase gradient elution, UV detection method is developed for the simultaneous determination of mono (2-ethylhexyl) phthalate [MEHP] and di (2-ethylhexyl) phthalate [DEHP] in tissue samples. The method is validated with respect to extraction recovery, inter and intra-day precision, linearity of response, detect ability, and specificity. The validated method has been successfully applied to the study of DEHP and MEHP in liver, kidney, testis, brain, and plasma samples from rat.

Introduction

Di (2-ethylhexyl) phthalate (DEHP) (Figure 1) is a commercially important plasticizer in polyvinyl chloride production, as a lubricant, and as a component of cosmetics such as nail polish and hair spray. Phthalates are not irreversibly bound in the polymer matrix and can migrate into the environment as a pollutant or into the body by transfer from storage materials to products such as food (1,2), infant formulas (3), or pharmaceutical products (4). Several investigations reported that DEHP was found in tissues of human patients who had received transfusions of blood stored in plastic bags or through medical devices (5–9).

Loff et al. (5) have quantified DEHP levels which have leached from various medical products such as PVC infusion tubing used in parenteral nutrition, blood transfusion products and pharmaceuticals used to care for neonatal children. Demore et al. (4) studied the release of DEHP from containers when the antineoplastic drug etoposide was stored. Etoposide was evaluated because it is prepared with the surfactant polysorbate 80, which is believed to release of DEHP from PVC containers. The potential health risks associated with DEHP leached from polyvinyl chloride medical devices has been recently reviewed by the FDA Center for Devices and Radiological Health (10). Furthermore, FDA Center for Devices and Radiological Health has recommended considering alternatives when high-risk procedures including hemodialysis, transfusion, enteral nutrition or total parenteral nutrition are to be performed on male neonates, pregnant women who are carrying a male fetus, and peripubertal males (11).

Given the high levels of production and ubiquitous exposure to phthalates, there are concerns that phthalates may be acting as toxicants, especially as an endocrine disruptor. This is an active area of toxicological research in both animals and humans with a sizeable literature demonstrating the potential of phthalates to act as carcinogens and testicular toxins (12–20). The important question is to relate toxic effects to actual exposure levels in various tissues.

Currently, there are several gas chromatographic (21–24) high-performance liquid chromatography (HPLC)–UV (6,25,26), and HPLC–mass spectrometry (MS) (27–29) methods to determine DEHP in various food and biological fluids and tissues. Most of these methods are specific for a certain matrix. The purpose of this study was to determine the reliability of a unified HPLC method (30) to simultaneously determine and quantify DEHP and its metabolite (MEHP) in blood, plasma, liver, brain, and testes. We will present the results of several validation parameters, which were tested.

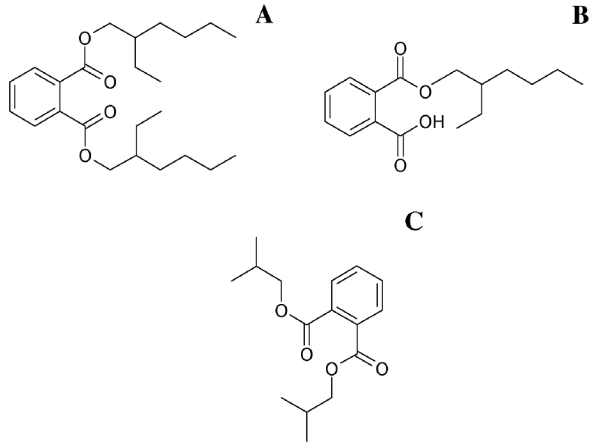

Figure 1. Chemical structures of DEHP (A), MEHP (B), and DIBP (C) (internal standard).

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Experimental

Instrumentation

Validation of the test method was performed on 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with G1313A autosampler, G1322A degasser, G1311A quaternary pump, and G1314A UV–vis detector. A second 1100 Series HPLC system (Agilent Technologies) with a diode array detector was used to test for intermediate precision on a different instrument. A ChemStation Data Acquisition System was used with both instruments.

The HPLC separation was performed with an Altima C_{18} Column (150 × 4.6 mm, 5 µm) and a pre-column filter from Alltech Associates Inc. (Deerfield, IL). A Polytron (Brinkmann Instruments, Westbury, NY) was used to homogenize the tissues and a Sonifier Cell Disruptor, (Branson Instruments Inc., Stanford, CT) was used to further disrupt the tissue samples.

Chemicals and Reagents

Phthalic acid mono-2-ethylhexyl ester, phthalic acid di-2-ethylhexyl ester, and phthalic acid diisobutyl ester (DIBP) were purchased from TCI America (Portland, OR). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific. The water used was purified using a Milli-Q gradient A10 system (Millipore Corp., Milford, MA). All other chemicals and solvents were obtained from commercial sources and were of high purity. The stock solutions of DEHP, MEHP, and Internal standard DIBP were prepared in 100% ACN.

Animals

Subjects were Holtzman Sprague-Dawley rats purchased from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed in polycarbonate cages with heat-treated chip bedding, and fed a standard rat diet (Purina 5012) and tap water. The animal room was on a 12/12 h lighting schedule and maintained at 70 ± 2 degrees over the course of the experiment. All animals used in these analyses were adults over 100 days of age. The animals were maintained and treated according to NIH guidelines for ethical treatment of research animals.

Sample preparation procedures

The organs were weighed and then homogenized using a Brinkmann polytron. Approximately 1 g of the chopped tissue sample was placed in a 10-mL reaction vial (for extraction) containing 7.5 mL of 100% ACN, 0.02 mL of 85% phosphoric acid and 0.3 g of NaCl. After adding 1.5 mL DIBP (concentration 256 µg/mL) as an internal standard into the extraction solution, homogenization of the tissue sample was carried for 10 min using a sonifier cell disruptor. The sample was then vortexed for 5 min and filtered using an Acrodisc CR 25 mm syringe filter before injection into the HPLC system.

For liquid samples such as plasma and milk, 200 µL of the sample was added into a glass tube, followed by 1 mL of 100% ACN, 4 µL of 85% phosphoric acid, and 200 µL internal standard. The solution was vortexed for 1 min then filtered to separate proteins and other insoluble substances using an Acrodisc syringe filter prior to injection into the HPLC system.

HPLC method

A reversed-phase HPLC method was used under a gradient elution range of 60% to 100% ACN with a gradient time of 5 min at a flow rate of 1 mL/min, then increased to 2 mL/min for 3 min while keeping the final solvent composition at 100% ACN. The acidity of the mobile phase was controlled at pH 3.0 with 25mM NaH_2PO_4. H_2O buffer. A 235 nm UV wavelength was used for detection. Applications of the method on spiked samples of rat liver, kidney, testis, brain, and plasma are shown in Figure 2.

Results and Discussion

Method validation

Systematic validation studies were conducted to determine how precisely and accurately the developed method can produce reliable and reproducible data when the developed method is applied in different environments such as different instruments or different laboratories. Several validation parameters such as precision (both repeatability and reproducibility), accuracy, limit of detection (LOD), limit of quantitation (LOQ), linearity, range, and robustness were examined.

Accuracy

Three preparations of five different concentration levels of standard solutions were made from a stock solution containing 35.4 µg/mL DEHP, 43.4 µg/mL MEHP, and 247 µg/mL DIBP (Table I). Similar amounts were also spiked into control samples of liver free from DEHP and MEHP. The samples were injected into the HPLC system, and the percent recovery was calculated (Table I). The percent recovery for MEHP at all concentration levels was above 100%. In the case of DEHP, the percent recovery increased from an average of 91.2% at 4.25 µg/mL to 99.1% at 24.78 µg/mL. Percent RSD, for both DEHP and MEHP, was found to be less than 1% under all concentrations studied except for 6.02 µg/mL DEHP, 7.39 µg/mL MEHP and 49.4 µg/mL DIBP where percent recoveries were comparatively high.

Precision

In this study, method precision (intra-assay repeatability), instrument precision (injection repeatability), and intermediate precision were investigated. To determine method precision (intra-assay), different concentrations of DEHP and MEHP were spiked into liver samples, which were free of DEHP and MEHP. The samples were analyzed by the same analyst and in the same day. Percent relative standard deviation were 1.61% (n = 6) and 0.89% (n = 6) for DEHP and MEHP, respectively.

To assess injection precision, a sample containing 5.45 µg/mL DEHP, 10.1 µg/mL MEHP, and 42.57 µg/mL DIBP was injected five times consecutively into the HPLC system. The calculated %RSD of the peak area (2.43%) was relatively high for DEHP in comparison to MEHP (0.34%) and DIBP (0.31%). This can be related to the lower concentration as well as longer retention time of DEHP.

Intermediate precision was also studied in order to determine the agreement of the measurements when the method is used on different days, within the same laboratory, using different HPLC systems, and conducted by different analysts. It also involved

multiple preparations of the sample. The method was tested using a different HP 1100 instrument from Agilent Technologies with a diode-array detector and on a different day. Known concentrations of DEHP and MEHP were spiked into six liver control samples free of DEHP and MEHP, and the samples were extracted and analyzed. The %RSD was less than 5% (n = 6) for both DEHP and MEHP.

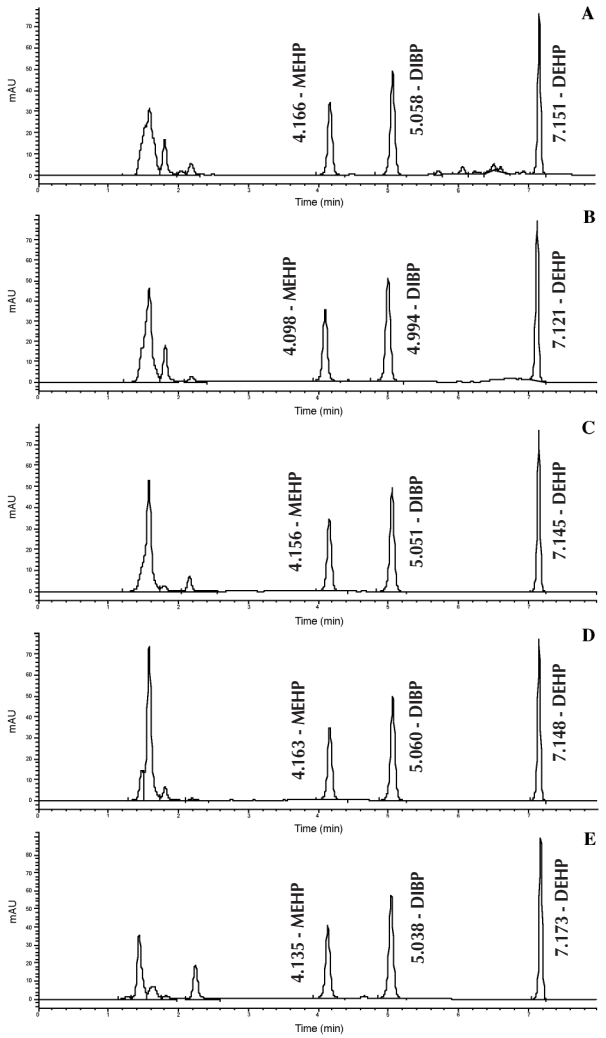

Figure 2. Effect of rat sample matrix on the separation of DEHP, MEHP and DIBP; liver (A), kidney (B), testis (C), brain (D), plasma (E). Condition: 60% to 100% ACN for 5 min at flow rate of 1 mL/min and at 100% ACN for 3 min at flow rate of 2 mL/min; buffer salt: 25mM $NaH_2PO_4.H_2O$, pH 3.0.

Table I. Accuracy Studies

Concentration (mg/mL)			% Recovery	
DEHP	MEHP	DIBP	DEHP	MEHP
4.25	5.21	49.40	91.2 ± 0.8	107.1 ± 0.4
6.02	7.39	49.40	95.6 ± 2.3	106.4 ± 1.6
8.14	9.98	49.40	99.9 ± 0.1	105.9 ± 0.6
16.28	19.96	49.40	97.5 ± 0.3	101.1 ± 0.2
24.78	30.38	49.40	99.1 ± 0.4	101.8 ± 0.3

Linearity

A stock solution containing 35.4 µg/mL DEHP and 43.4 µg/mL MEHP was sequentially diluted with 100% ACN in order to prepare several standard solutions of which the lowest concentration is the limit of quantitation (Table II). Similar concentrations of the standard solutions were prepared by spiking liver samples (free of DEHP and MEHP) with fixed amounts of DEHP and MEHP prior to extraction. In both cases, all standard solutions were spiked with a certain amount of the internal standard (10 mL of 247 µg/mL of DIBP). Standard solutions prepared in 100% ACN as well as in liver control samples free of DEHP and MEHP were injected into the HPLC.

Two calibration curves, (one for standard and another for spiked liver samples) were constructed by plotting concentration ratio versus peak area ratio of the analyte over the internal standard at different concentration levels (Figure 3). Both curves are linear within the range of concentration studied. In addition, it passes through the origin. This indicates that all replicate measurements, at each concentration level, are equivalent and the quantification can be performed using a single point calibration approach. Furthermore, the slopes and correlation coefficients for DEHP and MEHP are similar under both conditions. This also confirms a complete recovery.

LOD and LOQ

LOD and LOQ were measured by successively diluting a stock solution containing 137 µg/mL DEHP, 57 µg/mL MEHP, and 191 µg/mL DIBP with 100% ACN prior to HPLC injection. LOD and LOQ for DEHP were found to be 1.37 µg/mL and 4.8 µg/mL, respectively. For MEHP, LOD and LOQ were found to be 0.57 µg/mL and 2.39 µg/mL, respectively. These conclusions are based on the results of the concentrations that correspond to signal-to-noise ratios of 3 and 10 for LOD and LOQ, respectively. The % RSD of peak ratio response for four replicate injections of six individual preparations at LOQ was found to be less than 1%.

Range

The range of the developed method was tested to determine the lowest and the highest concentrations where the method has high accuracy and precision as well as a good linearity. In addition, it should cover all levels for the routine analysis. Based on

Table II. Linearity Studies in Method Validation

Concentration (mg/mL)			% Recovery	
DEHP	MEHP	DIBP	DEHP	MEHP
0.46	0.56	49.40	N/D*	163.0 ± 3.3
0.81	1.00	49.40	74.9 ± 1.9	130.5 ± 0.8
2.00	2.47	49.40	98.8 ± 4.1	130.1 ± 0.8
4.25	5.21	49.40	91.2 ± 0.8	107.1 ± 0.4
6.02	7.39	49.40	95.6 ± 2.3	106.4 ± 1.6
8.14	9.98	49.40	99.9 ± 0.1	105.9 ± 0.6
16.28	19.96	49.40	97.5 ± 0.3	101.1 ± 0.2
24.78	30.38	49.40	99.1 ± 0.4	101.8 ± 0.3

* N/D = not detectable.

the result of linearity, precision, and accuracy, the method appears to be linear from 0.81 µg/mL to 24.78 µg/mL for DEHP and from 1 µg/mL to 30.38 µg/mL for MEHP.

Robustness

To examine the robustness of the method toward the initial composition of the gradient (%B_i), a sample containing 5.45 µg/mL DEHP, 10.10 µg/mL MEHP, and 42.57 µg/mL DIBP was injected at the nominal %B_i of 60% and ± 5%. No significant changes in retention times of DEHP, MEHP, and DIBP were observed. Similar results were obtained when changing the mobile phase flow rate by ± 0.25 mL/min. The stability of DEHP, MEHP, and DIBP over time under the laboratory conditions was studied in 100% ACN and in liver extract. The results revealed that after six days under laboratory light at 25°C, no significant changes in peak area were observed. It confirms that no sample protection is necessary to prevent degradation when used for more than 24 h.

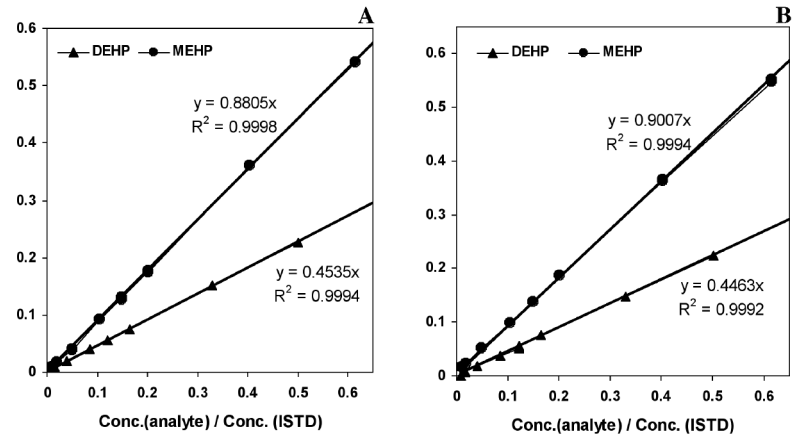

Figure 3. Linearity plot of peak area ratio versus concentration ratio of analytes over internal standard (ISTD) at different concentration levels for standard solutions in 100% ACN (A) and liver extract (B).

Table III. Determination of DEHP and MEHP in Biological Samples Using Standard Solutions in 100% ACN and Different HPLC System

Sample Type	Instrument I		Instrument II				
	Amount (mg/g organ)*		Peak Purity			Amount (mg/g organ)*	
	DEHP	MEHP	DEHP	MEHP	DIBP	DEHP	MEHP
Liver	12.9 ± 0.9	127.0 ± 2.1	999.77	992.83	999.21	35.9 ± 0.4	134.8 ± 0.1
Kidney	N/D†	20.7 ± 0.1	–‡	999.81	999.62	6.5 ± 0.1	21.2 ± 0.3
Testis	N/D	7.5 ± 0.1	N/D	999.94	999.68	N/D	7.9 ± 0.1
Brain	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Plasma	21.9 ± 0.5	77.1 ± 3.0	999.74	999.74	999.72	24.5 ± 1.9	81.7 ± 1.8

* mg/mL in the case of plasma sample.
† N/D = Not detectable.
‡ Not enough data for purity calculations available.

Table IV. Storage Stability of Grinded Organ Samples at 4°C for 12 h Prior to Extraction

Sample Type	Peak Purity			Amount (mg/g organ)	
	DEHP	MEHP	DIBP	DEHP	MEHP
Liver	999.29	999.76	999.44	29.9 ± 7.0	129.8 ± 1.1
Kidney	–*	999.81	994.86	6.8 ± 1.5	24.1 ± 2.1
Testis	N/D†	999.89	999.64	N/D	7.9 ± 0.1
Brain	N/D	N/D	N/D	N/D	N/D

* – Not enough data for purity calculations available
† N/D = Not detectable

Method Applications

The validated method was applied in different biological samples such as liver, kidney testis, brain, and plasma to determine the concentration of DEHP and MEHP by a HPLC system (Instrument I). A single point calibration approach was used. Tissue samples were collected 3 h after an oral dose of 1000 milligram per kilogram body weight of DEHP dissolved in olive oil. The results clearly indicate a complete conversion of DEHP to its metabolite MEHP in most of the samples after only 3 h (Tables III). In addition, DEHP as well as MEHP were found in both liver and plasma samples while only MEHP was observed in kidney and testis samples. Brain sample did not show any level of DEHP or MEHP. Furthermore, high concentrations of MEHP were found in both liver and plasma samples.

The collected samples, from different rat organs, were also analyzed on a different HPLC system (Instrument II) equipped with diode array UV detector to check for purity of the peaks of DEHP, MEHP, and DIBP. The results confirmed no interferences in the peaks of interest from sample matrices.

Finally, the role of storing ground organ samples overnight at 4°C was investigated. The samples were tested on the following day using a standard solution of 100% ACN and a single point calibration approach. The reliability and reproducibility of these results indicate that keeping ground tissue samples at 4°C (Table IV) does not lead to significant changes.

Conclusions

The proposed reversed-phase HPLC method was validated for the determination of DEHP and its metabolite (MEHP) in dif-

ferent biological samples. The developed method gave acceptable method precision (< 5.00% RSD) when analyzed by different analysts, on a different day, and using different HPLC systems. A recovery of more than 95% of phthalate esters were obtained from the spiked liver samples at five different concentrations between 4.25 and 24.78 µg/mL. Both DEHP and MEHP show a linear detection response with a coefficient of variation more than 0.999. The developed method is relatively insensitive to small changes in experimental condition, which confirms its robustness. The validated method has been successfully applied to study DEHP and MEHP in liver, kidney, testes, brain, and plasma samples of rat. The results confirm its reliability. The results of animal studies also indicate that DEHP is converted to its metabolite MEHP in liver, kidney, testis, and plasma of the rat.

References

1. K. Clark, I.T. Cousins, D. Mackay, and K. Yamada. Observed concentrations in the environment. In: C.A. Staples, (Eds.), *The Handbook of Environmental Chemistry.* Springer-Verlag, New York, 2003, pp. 125–77.
2. Y. Tsumura, S. Ishimitsu, A. Kaihara, K. Yoshii, Y. Nakamura, and Y. Tonogai. Di(2-ethylhexyl) phthalate contamination of retail packed lunches caused by PVC gloves used in the preparation of foods. *Food Addit. Contam.* **18:** 569–79 (2001).
3. J.H. Petersen and T. Breindahl. Plasticizers in total diet samples, baby food and infant formulae. *Food Addit. Contam.* **17:** 133–41 (2000).
4. B. Demore, J. Vigneron, A. Perrin, M.A. Hoffman, and M. Hoffman. Leaching of diethylhexyl phthalate from polyvinyl chloride bags into intravenous etoposide solution. *J. Clin. Pharm. Ther.* **27:** 139–42 (2002).
5. S. Loff, F. Kabs, K. Witt, J. Sartoris, B. Mandl, K.H. Niessen, and K.L. Waag. Polyvinylchloride Infusion Lines Expose Infants to Large Amounts of Toxic Plasticizers. *J. Pediatr. Surg.* **35:** 1775–81 (2000).
6. K. Kambia, T. Dine, B. Gressier, A.F. Germe, M. Luyckx, C. Brunet, L. Michaud, and F. Gottrand. High-performance liquid chromatographic method for the determination of di(2-ethylhexyl) phthalate in total parenteral nutrition and in plasma. *J. Chromatogr. B Biomed. Sci. Appl.* **755:** 297–303 (2001).
7. K. Kambia, T. Dine, B. Gressier, S. Bah, A.F. Germe, M. Luyckx, C. Brunet, L. Michaud, and F. Gottrand. Evaluation of childhood exposure to di(2-ethylhexyl) phthalate from perfusion kits during long-term parenteral nutrition. *Int. J Pharm.* **262:** 83–91 (2003).
8. C. Buchta, C. Bittner, P. Hocker, M. Macher, R. Schmid, C. Seger, and M. Dettke. Donor exposure to the plasticizer di(2-ethylhexyl)phthalate during plateletpheresis. *Transfusion* **43:** 1115–20 (2003).
9. S. Loff, F. Kabs, U. Subotic, T. Schaible, F. Reinecke, and M. Langbein. Kinetics of diethylhexyl-phthalate extraction From polyvinylchloride-infusion lines. *J. Parenter. Enteral. Nutr.* **26:** 305–309 (2002).
10. Center for Devices and Radiological Health, US Food and Drug Administration, 2001, http://www.fda.gov/cdrh/ost/dehp-pvc.pdf.
11. Center for Devices and Radiological Health, US Food and Drug Administration, 2002, http://www.fda.gov/cdrh/safety/dehp.html.
12. L.G. Parks, J.S. Ostby, C.R. Lambright, B.D. Abbott, G.R. Klinefelter, N.J. Barlow, and L.E. Gray Jr. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences* **58:** 339–49 (2000).
13. J.D. Park, S.S.M. Habeebu, and C.D. Klaassen. Testicular toxicity of di-(2-ethylhexyl)phthalate in young Sprague–Dawley rats. *Toxicology* **171:** 105–15 (2002).
14. M. Dalgaard, C. Nellemann, H.R. Lam, I.K. Sorensen, and O. Ladefoged. The acute effects of mono(2-ethylhexyl)phthalate (MEHP) on testes of prepubertal Wistar rats. *Toxicology Letters* **122:** 69–79 (2001).
15. C.M. Carruthers and P.M.D. Foster. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Research (Part B)* **74:** 277–85 (2005).
16. J. Borch, M. Axelstad, A.M. Vinggaard, and M. Dalgaard. Diisobutyl phthalate has comparable anti-androgenic effects to di-*n*-butyl phthalate in fetal rat testis. *Toxicology Letters* **163:** 183–90 (2006).
17. C. Voss, H. Zerban, P. Bannasch, and M.R. Berger. Lifelong exposure to di-(2-ethylhexyl)-phthalate induces tumors in liver and testes of Sprague–Dawley rats. *Toxicology* **206:** 359–71 (2005).
18. B.A. Mangham, J.R. Foster, and B.G. Lake. Comparison of the hepatic and testicular effects of orally administered Di(2-ethylhexyl) phthalate and dialkyl 79 phthalate in the rat. *Toxicol. Appl. Pharmacol.* **61:** 205–14 (1981).
19. A.H. Mann, S.C. Price, F.E. Mitchell, P. Grasso, R.H. Hinton, and J.W. Bridges. Comparison of the short-term effects of di(2-ethylhexyl) phthalate, di(*n*-hexyl) phthalate, and di(*n*-octyl) phthalate in rats. *Toxicol. Appl. Pharmacol.* **77:** 116–32 (1985).
20. L.E. Gray, N.J. Barlow, J.R. Furr, J. Brock, M.J. Silva, D.B. Barr, and J.S. Ostby. Lifelong exposure to di-(2-ethylhexyl)-phthalate induces tumors in liver and testes of Sprague–Dawley rats. *The Toxicologist* **72:** 283 (2003).
21. N.P. Ching, G.N. Jham, C. Subbarayan, D.V. Bowen, A.L. Smit, C.E. Grossi, R.G. Hicks, F.H. Field, and T.F. Nealon. Gas chromatographic-mass spectrometric detection of circulating plasticizers in surgical patients. *J. Chromatogr. B* **222:** 171–77 (1981).
22. L.S. Hillman, S.L. Goodwin and W.R. Sherman. Identification and measurement of plasticizer in neonatal tissues after umbilical catheters and blood products. *N. Engl. J. Med.* **292:** 381–86 (1975).
23. R.J. Jaeger and R.J. Rubin. Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. *N. Engl. J. Med.* **287:** 1114–18 (1972).
24. P. Sjoberg and U. Bondesson. Determination of di(2-ethylhexyl) phthalate and four of its metabolites in blood plasma by gas chromatography–mass spectrometry. *J. Chromatogr. B* **344:** 167–75 (1985).
25. G.M. Pollack, J.F. Buchanan, R.L. Slaughter, R.K. Kohli, and D.D. Shen. Circulating concentrations of di(2-ethylhexyl)phthalate and its de-esterified phthalic acid products following plasticizer exposure in patients receiving hemodialysis. *Toxicol. Appl. Pharmacol.* **79:** 257–67 (1985).
26. H. Shintani. Pretreatment and chromatographic analysis of phthalate esters, and their biochemical behavior in blood products. *Chromatographia.* **52:** 721–26 (2000).
27. S. Takatori, Y. Kitagawa, M. Kitagawa, H. Nakazawa, and S. Hori. Determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in human serum using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **804:** 397–401 (2004).
28. R. Preuss, H.M. Koch, and J. Angerer. Biological monitoring of the five major metabolites of di-(2-ethylhexyl) phthalate (DEHP) in human urine using column-switching liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* **816:** 269-280 (2005).
29. M.J. Silva, N.A. Malek, C.C. Hodge, J.A. Reidy, K. Kato, D.B. Barr, L.L. Needham, and J.W. Brock. Improved quantitative detection of 11 urinary phthalate metabolites in humans using liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry. *J. Chromatogr. B* **789:** 393–404 (2003).
30. D. Sircar, S.J. Albazi, Y. Atallah, and W. Pizzi. Unified reversed-phase method for the determination of di (2-ethylhexyl) phthalate and its major metabolite, mono (2-ethylhexyl) phthalate, in biological samples. *J. Liq. Chrom. Relat. Tech.* **29:** 2931–2942 (2006).

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