Supplemental Material

Title: BPA, BPA glucuronide, and BPA sulfate in midgestation umbilical cord serum in a Northern and Central California Population

Roy R. Gerona [1], Tracey J. Woodruff[2], Carrie A. Dickenson [2], Janet Pan[2], Jackie M. Schwartz[2], Saunak Sen[3], Matthew M. Friesen [1], Victor Y. Fujimoto [1], Patricia A. Hunt*

1 Department of Laboratory Medicine, University of California, San Francisco
2 Program on Reproductive Health and the Environment, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco
3 Department of Epidemiology and Biostatistics, University of California, San Francisco
4 School of Molecular Biosciences and Center for Reproductive Biology, Washington State University

*Corresponding Author: Tracey J. Woodruff, woodrufft@obgyn.ucsf.edu, phone: (510) 350-1240

Total number of pages (including cover page): 8
Page S2-S4: Section S1
Page S5: Table S1
Page S6: Table S2
Pages S7: Figure S1
Page S8: Figure S2

Section 1. Environmental Chemical Analysis Methods

Chemicals. Bisphenol A and bisphenol A-d16 were obtained from Sigma (St. Louis, MO). BPA glucuronide and BPA sulfate were generously provided by the National Institute of Environmental Health Sciences (NIEHS). BPA-free water was purchased from Aqua Solutions (Deer Park, TX) while analytical grade methanol was acquired from Honeywell Burdick and Jackson (Muskegon, MI). Stock solutions of BPA standards were prepared at 1 mg/mL, aliquoted to 1 mL portions in amber vials and stored at -80°C. All calibration standards ranging in concentration from 0.1 to 80 ng/mL were prepared from the stock solution by serial dilution with double charcoal stripped human serum that was previously tested to be BPA free. Synthetic human serum was obtained from UTAK Laboratories Inc.

Solid Phase Extraction. Solid phase extraction was used to prepare serum samples prior to LC-MS/MS analysis. We tested the Water Oasis HLB SPE cartridge to determine if BPA glucuronide and BPA sulfate can be efficiently co-extracted from serum with BPA. All three analytes are efficiently captured by the column. Elution of each analyte, however, depended on the concentration of methanol in the eluting solution. By systematically collecting eluate fractions at increasing concentrations of methanol, BPA glucuronide and BPA sulfate were eluted off the SPE column between 20-80% methanol with peak elution at 50-60% methanol. BPA, on the other hand, eluted off the column between 40-100% methanol with peak elution at 70-80%. Hence, to completely elute all three analytes, pure methanol was used as eluting solvent in sample preparation. Recoveries between 86.5-98.5% were obtained using this solvent for elution (see Method section in main text). A solvent wash of 5% methanol cleans up the column with non-specific materials prior to analyte elution.

LOQ, LOD Determination. Method detection limit for each analyte was assessed by running a series of calibration standards (0.01-100 ng/mL), established as the lowest concentration of the analyte that gives a signal/noise ratio (S/N) of 3. The limit of quantification was established as the lowest concentration with S/N=10 while keeping the linear regression coefficient of the standard curve ≥ 0.95. All three analytes have LOQs of 0.1 ng/mL (part per billion). BPA and BPA glucuronide have LODs of 0.05 ng/mL while BPA sulfate has a LOD of 0.025 ng/mL. BPA sulfate at the lower end has a signal that is not linear, it abruptly decreases in intensity. Hence, its LOD is just 2X lower than BPA and BPA glucuronide. The LOD is calculated as the lowest concentration that gives a S/N≥ 3

Method Validation. To validate the method, its performance was assessed in terms of its precision, linearity, and recovery. Precision and recovery studies were conducted by running three different concentrations of analytes (low=0.5 ng/mL, medium=5 ng/mL, high=10 ng/mL) spiked into the matrix blank. Five samples of each concentration were run within a batch to calculate within-run precision and three batches of 5 samples of each concentration were run on three separate days to calculated between-run precision. Recovery for each sample run was calculated along with precision. The linearity study was done by running the calibration standards (0.1-80 ng/mL) five separate times on separate days and assessing the linear regression coefficient for the calibration plot for each analyte in each run.

Field Blank and IV Equipment Testing. We tested all equipment and materials used in sample collection and BPA processing using an appropriate blank. Details of the materials and blanks used along with results of the testing are described in Supplemental Table S2. Each of the field blanks are run in the materials and equipment used in the study in exactly the same procedure as

actual samples (see Supplemental Material, Table S2). Details of the IV equipment testing is described below.

We used two different approaches to determine if IV use during the procedure could contaminate our samples. First, we estimated the length of IV use for each patient using information abstracted from medical records. We then compared estimated length of IV use to the levels of BPA measured in umbilical cord blood using Spearmen correlation test, pearson correlation test, and linear and tobit regression. BPA analyte levels were $\log_{10}$ transformed for linear regression. Second, we tested both the IV fluid and the equipment used to place the IV for BPA. Samples of the IV fluid bag, capillaries and needles were gathered and assembled to mimic IV fluid delivery. IV fluid was allowed to drip at 2mL/min for 120 min. About 2mL of fluid was collected at 0, 15, 30, 60 and 120 min time points, approximates of the common lengths of time that patients receive IV fluid prior to pregnancy termination procedure. Each sample was then processed using the standard sample extraction protocol used in the study to analyze umbilical cord serum. Each extract obtained from the samples was run at the LC-MS/MS to measure BPA, BPA glucuronide and BPA sulfate.

Stability Study. We conducted stability studies on BPA, BPA glucuronide and BPA sulfate and found that the analytes are stable within six months at -80°C at 1 ug/mL (concentration of our stock solutions for our calibration curves).

Researchers who would like to repeat our protocol or have questions regarding analysis methods, please contact the corresponding author: Dr. Tracey J. Woodruff - woodrufft@obgyn.ucsf.edu, or Dr. Roy R. Gerona - roy.gerona@ucsf.edu)

Table S1. Steps in the collection and analysis of blanks to evaluate potential sources of BPA contamination, Northern and Central California, 2010-2012.

	Control Material			Analytical Result
	Solvent (10% MeOH)	BPA-free Serum extracted	BPA-free Serum extracted after simulated sample collection	
Sample Collection IV Fluid bag assembly, Gloves, Laminaria, Test Tube, Cryovial Tube	-	-	YES	**No BPA signal detected**
Sample Extraction Pipette tips, Test tube, Oasis HLB cartridge, Conical tube, Sample Vial, Water, Methanol	-	YES	YES	**No BPA signal detected**
LC Injection needle, Injection port, Column, Capillaries, Mobile phase solvents, Mobile phase reservoir	YES	YES	YES	**No BPA signal detected**
Mass Spectrometer Injection valve, Ion source, Quadrupoles, Collision Cell, Quadrupole, Detector	YES	YES	YES	**No BPA signal detected**

Sample Collection: Corning 2ml cryovial storage tube (Corning No.430488), IV bags plasma lyte (Baxter 2B2534, NDC 0338-0179-04)

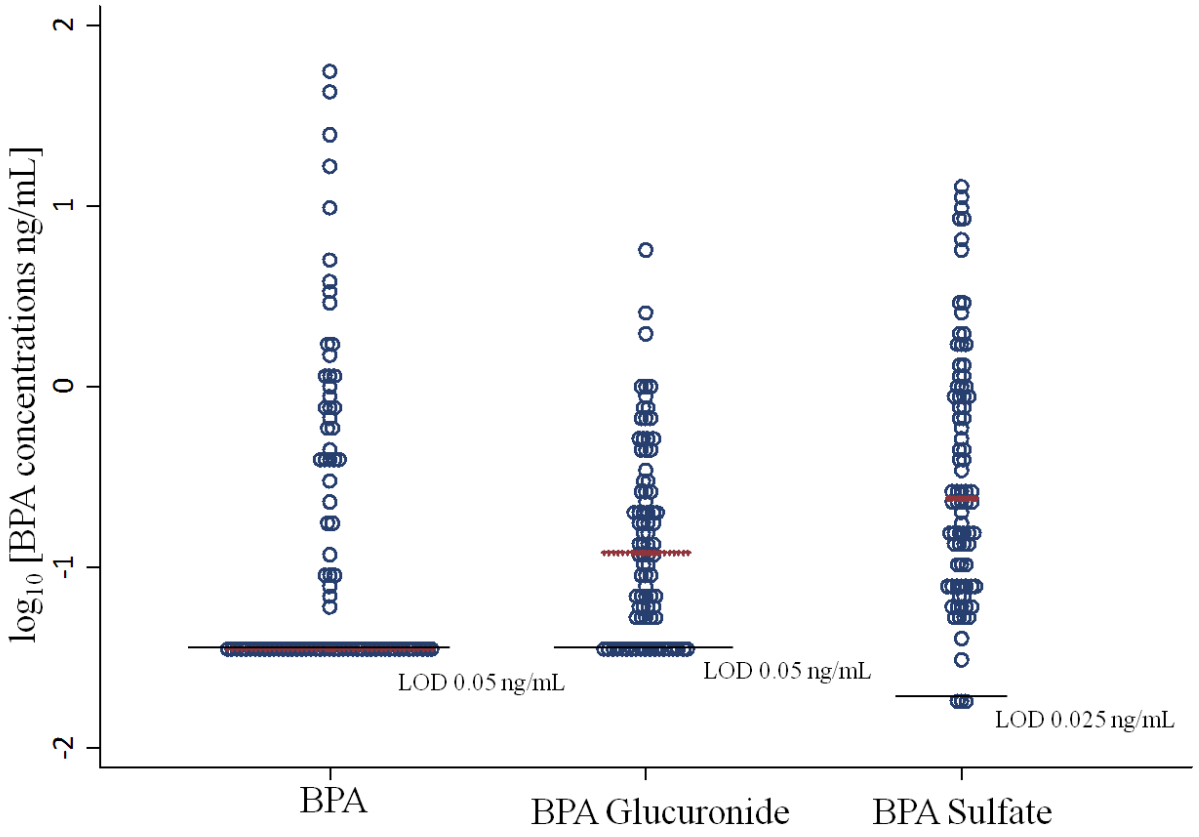
Sample Extraction: Fisher FinnTip 1000ul (REF 9401110); Fisher FinnTip Extended 1000uL (REF 9401420); Fisher Redi-Tip 1-200uL (REF 02707501); VWR 16X100mm Test Tubes (REF 60825-425); Kimble and Chase 13x110mm Conical Tubes (REF 73785-5); Agilent 2 mL Amber screw-capped vial (REF 5183-2069); Agilent Blue screw cap (REF 5182-0723); Waters Oasis HLB cartridge, 1cc, 10mg (REF 18600383)

Chromotography: Honeywell B&J Methanol (REF BJ230-4); Aqua Solutions Ultra Pure Water , HPLC grade, (BPA free REF W1089-10L); Corning 1000mL Media Storage Bottle (REF1395-1L); Agilent Extend C18 column, 1.8um, 4.6X100mm (REF 89013-926)

Table S2. Detection Frequency and Geometric Mean (GM) levels (ng/mL) of BPA, BPA Glucuronide, and BPA Sulfate in Umbilical Cord Serum, by Infant Sex and Gestational Age, Northern and Central California, 2010-2012 (n=85).

	BPA		BPA Glucuronide		BPA Sulfate	
	% >LOD	GM	%>LOD	GM	%>LOD	GM
Infant Sex						
Females (n=44)	45	0.16	84	0.17	98	0.31
Males (n=24)	46	0.15	84	0.14	92	0.27
Unknown (n=17)	53	0.15	53	0.09	100	0.42
Gestational Age						
< 20 weeks (n=22)	45	0.18	36	0.16	95	0.27
20-22 weeks (n=30)	40	0.13	87	0.15	97	0.28
> 22 weeks (n=33)	55	0.16	70	0.12	97	0.40

Figure S1. Distribution of Log_{10}-Transformed BPA, BPA Glucuronide, and BPA Sulfate Analyte Levels in Mid-gestation Umbilical Cord Serum, Northern and Central California, 2010-2012 (n=85).



[a] Red lines denote median concentrations

Figure S2. Total BPA Composition across quintiles of Total BPA, Northern and Central California, 2010-2012 (n=85).