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Application of a novel mass spectrometric (MS) method to examine exposure to Bisphenol-A and common substitutes in a maternal fetal cohort

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ABSTRACT

The use of Bisphenol A (BPA) has widely been replaced in consumer products by analogs BPB, BPE, BPF, BPS, and BPAF. Recent studies have linked these substitutes to similar adverse health outcomes as BPA, including disruption of endocrine pathways in animal and human studies. We designed a novel MS method, developed specifically for this study, to capture the most relevant BPA alternatives, BPB, BPE, BPF, BPS, BPAF and 4-NP in human blood and urine to quantify potential in utero exposures. To our knowledge, this is the first study to explore in utero exposure to these BPA analogs and the first U.S. study to test for BPA in maternal/fetal pairs. The method was run on 30 paired maternal urine and fetal cord blood samples from mothers undergoing elective Caesarean sections. 90% of mothers and 77% of babies tested positive for at least one BP analog. 83% of mothers tested positive for BPAF, 60% for BPS, 57% for BPB, 17% for BPF and 7% for BPA. 57% of babies tested positive for BPAF and 50% for BPF. BPA and BPB were detected in one cord blood sample each. BPS was not detected in cord blood. BPE was not detected in any fetal cord blood or maternal urine samples. These findings demonstrate the pervasiveness of some BP analogs in pregnant women and their babies at birth.

Abbreviations: BPA, bisphenol A; BPB, bisphenol B; BPE, bisphenol E; BPF, bisphenol F; BPS, bisphenol S; BPAF, bisphenol F; DIEHC, Deirdre Imus Environmental Health Center®; HIPAA, Health Insurance Portability and Accountability Act; MDL, method detection limit; PHI, protected health information; 4-NP, (4-nonylphenol)

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Introduction

In its 2008–09 annual report, the President's Cancer Panel stated that "exposure to potential environmental carcinogens is widespread. One such ubiquitous chemical, bisphenol A (BPA), is still found in many consumer products and remains unregulated in the United States, despite the growing link between BPA and several diseases, including various cancers" (The Presidents Cancer Panel 2010). Nearly a decade later, and after more than 75 human studies on environmental levels of BPA in children and adults, health concerns about this chemical persist among consumers as well as the medical and scientific communities (Rochester 2013). BPA is a common ingredient in polycarbonate plastic and epoxy resins used to produce products as diverse as food packaging, thermal receipts, dental sealants, and historically was used to make baby bottles and water bottles. In July 2012, The Food and Drug Administration (FDA) amended the food additive regulations "to no longer provide for the use of polycarbonate (PC) resins in infant feeding bottles (baby bottles) and spill-proof cups…because these uses have been abandoned" (Federal Register/Food and Drug Administration 2012). In July 2013, the FDA again amended regulations to "no longer provide for the use of BPA-based epoxy resins as coatings in packaging for infant formula" (Federal Register/Food and Drug Administration 2013. Canada banned the sale of polycarbonate baby bottles containing BPA in 2010 as did the European Union in 2011 (Government of Canada 2010; The European Commission 2011).

In recent years, consumer product manufacturers have therefore substituted BPA with other BP analogs (BPXs), or "cousin chemicals," that are structurally similar to their predecessor compound. Studies have shown that several of these analogs are perhaps no safer than the BPA they replace. They have similar estrogen-mimicking properties and links to adverse health outcomes like those produced by BPA, including disruption of endocrine pathways and altered hormonal status in both animal and human studies (Cabaton et al. 2006; Rosenmai et al. 2014). For that reason, structural BP analogs used instead of BPA may also be termed "regrettable substitutions."

The widespread exposure of humans to various plasticizers and concern about environmental factors causing endocrine disruption and cancer in children has been well documented. BPA has been detected in over 90% of the U.S. population, with the highest concentrations reported in children (Calafat et al. 2008). A 2012 study conducted by Liao et al. showed that BPS was found in 81% of urine samples analyzed across the United States and seven Asian countries. The study results included concentrations ranging from below the limit of quantitation (LOQ; 0.02 ng/mL) to 21 ng/mL (geometric mean: 0.168 ng/mL). After Japan, the US had the second highest urinary BPS concentration (Liao et al. 2012b). Zhou et al. analyzed 100 U.S. samples of human urine from 2009–2012, finding BPA in 95% (median concentration (0.72 ng/mL), BPS in 78% (0.13 ng/mL), and BPF in 55% (0.08 ng/mL) (Zhou et al. 2014). Adrianou et al. found BPA and BPF in 100% of 212 urine samples collected in 2013–2015 from adult, non-pregnant women in Cyprus and Romania (Andrianou et al. 2016). Exposure sources included water consumption from plastic bottles, canned food, cleaning habits and use of personal care products including perfume, deodorant, cosmetics, shampoo, conditioner, body lotion, hair dye and nail polish (Andrianou et al. 2016). As our study population was also all female adult women, it is reasonable to expect that many of the exposure sources for BPA and its structural analogs were similar.

For both females and males, exposure sources for bisphenols have become ubiquitous around the globe and are present to varying degrees in personal products, food additives, detergents, medications, dental sealants, and an array of plastics, including baby bottles and children's toys (Andrianou et al. 2016; Calafat et al. 2008; Federal Register/Food and Drug Administration 2012; Federal Register/Food and Drug Administration 2013; Liao and Kannan 2014; Liao et al. 2012b; Rosenmai et al. 2014).We sought to quantify the presence of BPA and known analogs in fasting, term pregnant women undergoing elective Caesarean sections and their newborns through matched samples of maternal urine and umbilical cord blood. The presence of BPA has been explored in prenatal biological fluids, showing the chemical does cross the placenta and is found in cord blood (Ikezuki et al. 2002). Though BPA has been studied in human maternal/fetal pairs in Asia (Chou et al. 2011; Lee et al. 2008), this appears to be the first U.S. study. This is also the first known study to explore the presence of BP analogs in human mother-newborn dyads.

Methods

This research study explores the presence of endocrine-disrupting chemicals in 30 healthy maternal/fetal pairs at term (>37 weeks) Caesarean (non-laboring) delivery. We tested for the following in maternal urine and fetal cord blood: BPA, BPB, BPE, BPF, BPS, BPAF and 4-NP. Our team determined this list to be inclusive of the BP analogs most in use in commerce today (Alabi et al. 2014; Audebert et al. 2011; Cabaton et al. 2008; Cabaton et al. 2006; Cacho et al. 2012; Danzl et al. 2009; Gallart-Ayala et al. 2011; Gallart-Ayala et al. 2010; Grumetto et al. 2013; Higashihara et al. 2007; Liao and Kannan 2013; Liao and Kannan 2014; Liao et al. 2012a, c; Rastkari et al. 2010; Rochester and Bolden 2015; Rosenmai et al. 2014; Song et al. 2014; Sun et al. 2014; Wang et al. 2014; Yang et al. 2014a, b; Zhou et al. 2014).

To achieve a total of 30 evaluable samples, 35 patients were consented and enrolled. Five were omitted prior to analysis due to complications with collecting samples or failure to meet all study inclusion criteria (below). The samples were acquired at Caesarean section deliveries at Hackensack University Medical Center, Hackensack, New Jersey. Treatment of patients was not under investigation in this research study. Cord blood samples were collected from otherwise discarded umbilical cords/cord blood samples taken for routine newborn testing.

Inclusion criteria were healthy women aged 18–40 years old scheduled for planned Caesarean delivery, and able and willing to give written informed consent. Exclusion criteria were inability to provide informed consent, subjects undergoing an emergency Caesarean delivery or any vaginal delivery, subjects already planning to bank or donate cord blood for other reasons, and pregnancy-related complications or conditions that could possibly alter concentrations of the chemical exposures studied (including gestational diabetes, preeclampsia, preterm labor, prolonged rupture of membranes, active maternal infection).

The maternal urine sample was collected directly into a polypropylene plastic urine collection cup with high-density polyethylene lid, pre-screened for leachability of analytes under collection and storage conditions. Approximately 10 ml of urine was liquated from the urine collection container into two 5 ml polypropylene cryo-vials and stored at -80^{O}C until analysis.

Cord blood samples were extracted via syringe into vacutainers with no anti-coagulant and centrifuged for ten minutes at 2600 rpm at 18°C (room temperature). The serum was

then transferred to two 2 mL sterile polypropylene cryo-vials and stored immediately at -80°C. All lab supplies were analyzed prior to the start of the study to ensure they did not contribute exogenous bisphenols to the study samples. Once sample collection from all subjects was completed, the samples were shipped on dry ice to the Rutgers Environmental and Occupational Health Sciences Institute (EOHSI) for analysis, labeled with a coded identification number and date of collection.

Recruitment procedures & informed consent

During the study enrollment period (June-August, 2015), a member of the research team reviewed scheduled Caesarean deliveries to determine possible candidates. Patients were recruited after hospital admission for their procedure, at the time when consent is obtained for the Caesarean delivery. All mothers consented to the IRB-approved protocol and gave written, informed consent. Protocol review and approval was provided by the Institutional Review Board (IRB) of Hackensack University Medical Center, Hackensack, New Jersey.

Sample analysis

Sample analysis was performed at the chemical analysis facility core, part of the Environmental and Occupational Health Sciences Institute (EOHSI) at Rutgers University. BPA and their analogs BPXs (BPB, BPE, BPF, BPS, BPAF) and 4-NP were measured using an ultra performance liquid chromatography/mass spectrometry (UPLC/MS) technique. Specifically, a Thermo LTQ-XL mass spectrometer, interfaced to an Accela autosampler and Accela pump system was used for separation and quantitation of BPA analogs. A Hypersil Gold, 50 × 2.1 mm, 1.9 μm column with a 0.5 μm KrudKatcher Ultra filter guard column was used to separate the analytes. The separation was carried out using a gradient flow of water and acetonitrile.

Data were collected on a Thermo LTQ instrument using four separate MS segments each with between 1 and 4 scan events. The chromatographic elution order of the analytes; BPS, BPF, BPE, BPA, BPF, BPB, BPAF and 4-NP. Source fragmentation energy was 25V and normalized collision energies ranged between 30 and 60 and all ions were collected in negative mode. Electrospray ionization source was used to ionize the BPA analogs before introduction into the mass spectrometer. Capillary temperature was set at 350 degrees Celsius. A full description of precursor product ion pairs and other operating parameters is listed in Table 1 below.

This method was developed specifically for BPA alternatives using techniques previously employed in the development and implementation of other methods for quantification of

Table 1. BPX LC/MS analysis parameters.

Analyte	Precursor Ion $\left(\frac{m}{z}\right)$	Product Ion (s)	Segment	Collision Energy	Retention time (min)
BPS	249	108,156,185	1	60	1.35
BPF	199	93,105	2	50	2.41
BPE	213	198	2	50	3.04
BPA	227	213	2	59	3.39
BPB	241	212,226,265	3	40	3.87
BPAF	335	265	3	30	4.26
4-NP	219	106	4	50	7.41

analytes in biofluids (Brunetti et al. 2016; Winnik et al. 2009; Zhan et al. 2016), including those for BPA and its metabolites (Coughlin et al. 2011; Weinberger et al. 2013). The BPXs were purchased from Sigma Aldrich (St. Louis, MO) except BPB, which was purchased from TCI America (Portland, OR). An internal standard, 20 μl of D_{16}-BPA at 10 μg/ml (also Sigma Aldrich) was spiked into one ml of urine or serum to monitor for analyte recovery. To insure all (free and bound) BPA/analogs were captured, two fractions were analyzed in samples where BPA/analogs were detected; one containing total BPA/analog (including free, glucuronides and sulfates), the other a free fraction of BPA/analog only. The total concentration of BPA/analogs was measured after deconjugation of the sample using enzyme β-Glucuronidase, which also has a demonstrated sulfates activity. Specifically, 0.25 ml sodium acetate buffer (pH = 4.65) and 10 μl of β-Glucuronidase from *Helix pomatia* was added into 1ml of urine or serum. The enzymatic deconjugation was carried out in a water bath at 37°C overnight. The analytes were isolated from urine or serum using liquid-liquid extraction with 2 × 3 ml of dichloromethane followed by centrifugation and reconstitution in 50 μl of 50% methanol. The free BPA/analog fraction was measured in urine or serum samples using the same method as that for the total BPA/analogs (described here) but without addition of buffer and enzyme. The free fraction was used to determine the ratio of free/total to confirm that the bulk of the analytes were metabolites and that external contamination did not represent a significant fraction of the value reported.

Calibration curves were generated using standards generated in matrix and prepared exactly as samples. The method detection limit (MDL) was estimated based on the lowest standard that returned a signal to noise ratio of 2.5 or greater for Q2, the quantitation ion. A summary of the concentration ranges and MDLs, reproducibility and repeatability for all analytes is presented in Table 2 below. Because these methods are newly applied to these environmental contaminants currently being used to replace BPA, data are here reported as a frequency of detection and the ranges of the positive values. The meaning of absolute values with respect to their potential for endocrine disruption and other adverse health effects will require further investigations.

During protocol development, we included the testing of samples for 4-Nonylphenol (4-NP), a chemical used in the manufacture of some plastics. This new analytical method originally included 4-NP, requiring to compromise methodological conditions for both BPA and 4-NP. As a result, sensitivity for both of these compounds was lost and none of the

Table 2. Concentration ranges, MDLs, reproducibility and repeatability for analytes.

Cord blood Serum	BPS	BPF	BPE	BPA	BPB	BPAF	4NP
Day to day variation	5.06%	8.17%	2.57%	2.44%	5.29%	5.01%	11.6%
Within day variation	*27.0%	1.44%	0.20%	3.52%	5.85%	8.24%	15.7%
MDL (ng/ml) max**	0.5	2.5	1.9	1.7	0.5	0.14	10
Max Calibration Std	16.0	160	16.0	16.0	16.0	1.60	160
Min Calibration Std	0.50	1.00	1.00	2.00	0.50	0.10	10.0
Maternal Urine	BPS	BPF	BPE	BPA	BPB	BPAF	4NP
Day to day variation	6.69%	8.40%	9.81%	5.91%	7.19%	4.59%	11.14%
Within day variation	9.95%	20.2%	0.31%	18.1%	12.7%	8.68%	5.55%
MDL (ng/ml) max**	1.41	6.4	2.08	1.35	1.28	0.04	10
Max Calibration Std	16.0	160	16.0	16.0	16.0	1.60	160
Min Calibration Std	0.50	10.0	1.00	1.00	1.00	0.05	10.0

*driven by one observation

**Lowest reported concentration with S/N>3

samples had quantifiable amounts of 4-NP and therefore it was not discussed in further detail. For this reason, in the post analysis, we chose to focus on bisphenols only.

Statistical analyses

The main objective of the study was to evaluate urine and serum concentrations of BPA and BP analogs and to identify any correlations between concentrations in the mother/baby pairs. Characteristics of the patients were tested as follows: continuous variables are presented using the mean and standard deviation where values were normally distributed, and as the median and IQR where distributions were not normal. Concentrations of measurements from urine are standardized to creatinine. Categorical variables are presented as counts (%). Difference in continuous variables between mothers and babies were assessed using paired-sample *t*-tests or paired-sample Wilcoxon signed rank test based on normality of the data. All data analysis in this study was performed using the R statistical package (R Core Team 2012).

Analysis of correlations

The correlation between concentrations of BPA and BP analogs in cord blood and urine paired samples were examined by the Pearson's correlation coefficient or Spearman's rank correlation coefficient. Pearson's (rho) or Spearman's rank correlation (rho), as appropriate, and 95% confidence interval (CI) using the Fisher transformation were calculated between BPA and BP analogs. Rho values of 0.60-0.79 indicate high correlation, values of 0.40-0.59 moderate, and values of 0.20-0.39 weak or low correlations.

Regression analysis

Formal analysis took the form of regression models between mothers' and their babies' BPA and BP analogs, with the possible inclusion of other factors such as mother's age or babies' sex. For the analysis, nonlinear relationships between the variables were explored using transformations of the concentrations, such as the log transformation. For all analysis in this study, any $P < 0.05$ was considered statistically significant.

Results

In this study, all non-zero values were used for frequency of detection, even those below the MDL. We define MDL as the lowest reproducible standard used to create the calibration curve with appropriate dilution factor allied to the sample. A value below the MDL must have a signal to background (S/N) of 2.5 or greater, to be reported as a non-zero, or positive.

There were 27 urine samples (90%) with at least one positive chemical measurement in urine (which includes 10 values $\geq$ MDL), and 23 newborn subjects (77%) with at least one positive chemical measurement in fetal blood (including 5 values $\geq$ MDL).

Table 3 shows that 83% of maternal subjects had BPAF in urine, 60% had BPS in urine and 57% had BPB in urine. In all, of the reported values, the free fraction represented less than 20% of the total and only the total was reported.

For newborn subjects, 50% had BPF in blood and 57% had BPAF in blood.

There were no positive values of BPE in any of the urine samples, and only two positive values of BPA (one was $\geq$ MDL). There were no positive values of BPE and BPS in any of

Table 3. Number of positive values.

BPS	BPF	BPE	BPA	BPB	BPAF
Urine					
18	5	0	2	17	25
Blood					
0	15	0	1	1	17

the blood samples. Blood BPA and BPB each had positive values for only one blood sample. There were 18 positive values of urine BPS. The summary statistics of the positive values are shown in Table 4.

There appeared to be a lack of relationship between the various chemicals measured and between maternal urinary and fetal serum levels highlighted in Table 5 with small correlation coefficients in most cases. In the instances where the correlation coefficient is large >0.4 (numbers in bold, e.g. 0.72 between urine BPB and BPAF), they are due to one data point affecting the result.

Correlation coefficients between urine and blood chemicals are small, as seen in Table 6. Note that there is only one positive BPB and BPA measurement in the fetal blood samples and so correlations with blood BPB and blood BPA are not informative. There is only one positive blood BPB and BPA measurement, so correlations with these two measurements are not informative. In summary, Table 6 shows correlations between blood chemicals with urine chemicals while Table 5 separates out the blood chemicals (shows correlations between them) and similarly for the urine chemicals.

There was no relationship between maternal urinary or cord blood levels of the various chemicals and maternal age, weight or height, nor was their variation related to fetal gestational age, birth weight, or sex.

Table 7 shows the proportion of subjects that tested positive for each of the chemicals.

Discussion

We found that 90% of mothers tested positive for at least one BP chemical in their urine, as 83% tested positive for BPAF, 60% for BPS and 57% for BPB. At least one BP chemical was found in 77% of newborns' cord blood samples, with BPAF and BPF found in 57% and 50%, respectively. One analog, BPAF, was found in a majority of both urine and cord blood samples. Yet despite the presence of BPS and BPB in a majority of maternal samples, no detectable BPS and only one detection of BPB was found in cord blood. Paradoxically, low prevalence of BPF in maternal urine samples contrasts with higher prevalence in cord blood samples.

Nearly all mothers were exposed to one or more of the six bisphenols tested. Their fasting status suggests this number could very well be higher, as the half-life for BPA and related chemicals is relatively short. Their babies were exposed at significantly high rates as well.

Table 4. Summary statistics of positive urine BPS.

Urine BPS (ng/ml)	Minimum	1st quartile	Median	Mean (std dev)	3rd quartile	Maximum
	0.004	0.055	0.19	0.847(2.05)	0.784	8.883

Note: *LLO (lowest level of observance).

Table 5. Correlation coefficients between maternal urine levels of the chemicals measured and the cord blood of their neonates.

Urine	BPS	BPF	BPA	BPB	BPAF	Blood	BPF	BPA	BPB	BPAF
BPS	1.00									
BPF	0.01	1.00				BPF	1.00			
BPA	0.11	0.11	1.00			BPA	−0.06	1.00		
BPB	−0.03	0.42	−0.10	1.00		BPB	−0.02	−0.03	1.00	
BPAF	0.03	0.69	−0.04	0.72	1.00	BPAF	−0.08	0.09	−0.09	1.00

While the method of transfer from mother to baby is not clear, especially when the BP analog detected in the mother is not present in the baby or vice versa, the exposures alone give cause for concern over potential health effects.

Previous studies indicate that BPA was ubiquitously detected in 80–90% of adults spot tested (non-fasting). BPA has also been detected in maternal/fetal pairs in Taiwan and Korea. In both of these countries, maternal blood samples were compared to their newborns' umbilical cord blood (Chou et al. 2011; Lee et al. 2008). The Taiwanese study of 97 maternal/fetal pairs assessed newborns for lower birth weight, smaller size for gestational age, and high leptin and low adiponectin secretion. The study found a positive association of these with maternal BPA levels (Chou et al. 2011). In the Korean study of 300 maternal/fetal pairs, BPA levels "ranged from non-detectable to 66.48 microg/L in pregnant women and from non-detectable to 8.86 microg/L in umbilical cords" (Lee et al. 2008). In a dose-response study of rat maternal/fetal pairs, Takahashi et al. found that BPA rapidly crosses the placenta, and that BPA levels rapidly decrease in both maternal and fetal rats following acute exposure (Takahashi and Oishi 2000).

Only two of our maternal urine samples and one cord blood sample contained BPA. One possible explanation is that BPA is increasingly removed from commercial products by manufacturers now using BP analogs instead. Parents are being educated in the marketplace to look for "BPA-free" products, but are largely unaware that substitute chemicals used in place of BPA may have similar adverse health effects. Therefore, the decline in the use of BPA in some consumer goods may have led to minimal exposure among our healthy subjects, with commensurate increase in exposure to BP analogs.

Another possible explanation for low levels of BPA in our maternal and newborn samples may be accounted for by the minimum of 8 hours fasting time required prior to elective Caesarean delivery. Data from the 2003–2004 National Health and Nutrition Examination Survey showed that urine BPA concentrations of 1,469 adult subjects declined markedly within a 4.5–8.5 hour timeframe following exposure (Stahlhut et al. 2009). However, Stalhut et al. also found "fasting time did not significantly predict highest (>12 ng/mL) or lowest

Table 6. Correlation coefficients between urine and blood chemicals.

	Blood	
Correlation Coefficient	BPF	BPAF
Urine	BPS −0.02	−0.06
	BPF 0.01	−0.17
	BPA −0.08	−0.14
	BPB −0.09	−0.06
	BPAF 0.00	−0.13

Table 7. Proportions for each chemical testing positive.

BPS	BPF	BPE	BPA	BPB	BPAF
Urine					
0.60	0.17	0.00	0.067	0.57	0.83
Blood					
0.00	0.50	0.00	0.033	0.033	0.57

(below limit of detection) BPA levels." These findings suggest that non-food BPA exposure and/or accumulation in body fat may influence the levels detected (Stahlhut et al. 2009). Other BPA sources include the coatings on cash register receipts, residential water supply lines where PVC pipes are used, dental sealants, dust and air.

Both this study and Stahlhut et al.'s (Stahlhut et al. 2009), explored chronic, everyday exposures. In acute human dosing studies, Volkel and colleagues found that administration of 5 mg of d_{16}-bisphenol A reached peak levels in urine 80 minutes after oral administration, with a half-life of less than six hours. Detectible levels in urine were exclusively d_{16}-bisphenol A glucuronide form, as free d_{16}-bisphenol A was below the limit of detection in both urine and blood (Volkel et al. 2002). As a point of reference, a meta-analysis by Andra et al. reported BPA levels resulting from tests directly on consumer personal care and home cleaning products such as tub and tile cleaner, body wash, shampoo, lotion, soap and laundry detergent were <100 μg/g, with most items testing at <10 μg/g (Andra et al. 2015; Dodson et al. 2012). Despite the presence of BPA in these products, none of the product labels listed BPA (Dodson et al. 2012). Human exposure tests on urine from dermal contact with thermal paper receipts after using hand sanitizer found unconjugated BPA levels at $\sim$7 ng/mL in serum and $\sim$20 μg/g total BPA in urine within 90 minutes (Hormann et al. 2014).

In another dosing study where volunteers received 25 μg of BPA (estimated maximum human daily intake), Volkel et al. found that in the male and female subjects, 85% and 75% respectively of the exogenously administered BPA were detected as BPA-glucuronide within 5 hours after dosing (Volkel et al. 2005). A urinary half-life for elimination of approximately 4 hours was calculated from the excretion rates, similar to a previous study (Volkel et al. 2005).

While the relatively rapid clearance and short half-life of BPA may seem reassuring, the reported data and toxicokinetic models have generated debate. Reviewing these studies, in 2013 Vandenberg and colleagues found that current human exposure levels for BPA likely reflect the growing list of concerning health hazards found in laboratory animals (Vandenberg et al. 2013). They warn that the disparate levels of BPA reported in various studies is not as important as potential health effects from BPA. This was echoed from an earlier (2007) Vandenberg review showing that human exposure levels in serum, urine and other tissues are "within the range shown to cause effects in laboratory animals, and impact cell function in mechanistic studies in cell culture. Therefore, it is plausible and even likely that these levels are biologically active in humans, with obvious potential to cause disease or dysfunction" (Vandenberg et al. 2007). BPA is known in rat and mouse studies for affecting reproduction, the immune system, nervous system, and sexual differentiation in the brain as well as behavior, which is affected at levels below the human tolerable daily intake level (below 50 μg/kg) (Fernández et al. 2009; Kubo et al. 2003; Sawai et al. 2003). In a dosing study, MacLusky et al. found health affects in rats below the U.S. Environmental Protection Agency reference daily limit for human exposure dose of 50 μg/kg (MacLusky et al. 2005). Kubo et al.'s study involving maternal/fetal pairs suggests the effects of endocrine disruptors

are multigenerational (Kubo et al. 2003). Drake et al. found transgenerational effects may require contact with the environmental insult during key periods of growth and development (Drake et al. 2007). Though the molecular mechanisms in humans are unclear, these exposures may contribute to the origins of disease and other long-term effects by inducing permanent changes via epigenetic processes (Drake et al. 2007; Fernández et al. 2009). Inadera found that several BPA analogs, including BPAF and BPB had more estrogenic activity than BPA. He concluded that these estrogenic BPs may disrupt estrogen receptor-dependent pathways, therefore causing neurodevelopmental disorders (Inadera 2015).

Kitamura et al. studied BPA and 19 related compounds including four of the analogs included in our study: BPB, BPF, BPS and BPAF. Estrogenic activity was tested with the human breast cancer cell line MCF-7 cells, androgenic activity was tested with the mouse fibroblast cell line NIH3T3 and thyroid hormonal activity was tested with the pituitary cell line GH3 (Kitamura et al. 2005). The study found that BPA and BPB were highly estrogenic, while BPB, BPS and BPA were significantly anti-androgenic (Kitamura et al. 2005). In total, BPA, BPB, BPAF and BPF "exhibited both high estrogenic and anti-androgenic activities," suggesting that some analogs have multiple endocrine-disrupting activities. (Kitamura et al. 2005).

Health effects in newborns are of primary concern. They do not have the ability to detoxify with the same efficiency as adults (Alcorn and McNamara 2003) and they have a longer latency period from the time of exposure, during which diseases can develop in later years. Neonates and young infants have some ability to metabolize BPA (Calafat et al. 2009; Rubin et al. 2006), but generally have immature glucuronidation capacity necessary to metabolize BPA (Alcorn and McNamara 2003), which is the primary method by which adult humans metabolize BPA. Nishikawa et al. found that BPA is highly glucuronidated in the adult liver, and the resulting BPA-GA is an inactive, non-estrogenic metabolite. Though the mode of transport from mother to fetus is poorly understood, it was found that this BPA is reactivated in the fetus, creating concern for potential adverse health effects in newborns (Nishikawa et al. 2010). Rubin et al. found mice perinatally exposed to low levels of BPA both during gestation and again through lactation, exhibited altered brain sexual differentiation (Rubin et al. 2006).

In summary, BPA has been shown to be a potent endocrine disruptor in human and animal studies, and animal studies have shown carcinogenic potential for breast and prostate tissues (Rochester 2013; Seachrist et al. 2016). In vitro studies and in vivo animal research has shown that BPS and BPF have been found to be as hormonally active as BPA (estrogenic, antiestrogenic, androgenic, and antiandrogenic) with endocrine-disrupting effects (Rochester 2015). BPS and BPF also showed other potentially adverse health effects including "altered organ weights, reproductive end points, and enzyme expression" (Rochester 2015). BPE has been shown in an in vitro study to have potential to cause DNA damage (Rosenmai et al. 2014). BPE and BPF also were shown to increase corticosteroid levels which is concerning given increases in these during gestation are linked to development of health effects related to metabolic syndrome and behavior changes in adulthood (Inadera 2015; Rosenmai et al. 2014). BPS and BPF were found to most increase progestogen levels in the same study, whereas BPA had little effect. Rosenmai et al. reported that introduction of synthetic progestagen *in utero* to be associated with masculinization of female mice and feminization in male mice (reviewed in (Rosenmai et al. 2014). BPAF has been shown to have endocrine-disrupting activity, specifically significant estrogenic activity when tested in a human breast cancer cell line of MCF-7 cells (Kitamura et al. 2005).

There were 27 subjects with at least one positive chemical measurement in urine (which includes 10 values ≥ MDL), and 23 subjects with at least one positive chemical measurement in cord blood (including 5 values ≥ MDL) (Table 2). With respect to other BP analogs, BPAF, BPB and BPS were the predominant chemicals found in maternal urine samples, and BPAF and BPF in cord blood samples.

The fact that BPS levels in urine are not correlated with levels in fetal cord blood suggests either rapid elimination from the mothers' body due to a short half-life, placental xenobiotic efflux mechanisms preventing maternal-to-fetal transport, the mothers' ability to filter out this chemical, or some combination. Of note, five maternal urine samples demonstrated presence of BPB compared with 15 cord blood samples. These discrepancies might be explained by differences in maternal/fetal metabolism and detoxification mechanisms, however there were also differences in the sensitivity of the urine versus blood analysis methods for bisphenols.

Note that for the chemical pairs (BPF, BPB), (BPF, BPAF) and (BPB, BPAF) in urine, the high correlations, indicated in bold font in Table 3, are all due to one data point which is subject ID#11. Removing ID#11 reduces the correlations by a large amount. Otherwise, all the correlations between blood chemicals are small as shown in Table 5. Again, there does not appear to be any evident correlations between the variables. The small correlations suggest there is no clear (linear) relationship between the chemical concentrations. We also calculated the percentage of mothers who tested positive for each chemical and what percent of babies tested positive for each chemical in Table 6.

Limitations

The primary limitation of this study was the fasting status of subjects enrolled prior to Caesarean section, which likely limited the amount of bisphenols circulating in mothers' urine and babies' cord blood. Elective Caesarean sections require mothers to neither drink nor eat for at least eight hours prior to the procedure. We did not record fasting duration at the time of urine collection, but in all cases, mothers were instructed to refrain from eating or drinking for a minimum of 8 hours prior to their scheduled Caesarean. Given the concerns with consenting a woman in active labor, enrolling only Caesarean section patients allowed for efficient use of study staff and resources for this pilot study.

The limit of quantitation was different for each analyte and different for the free vs. total analyte. Deconjugation with β-Glucuronidase causes an increase in background (and the corresponding noise) for all analytes, even with an MS2 based method. In addition, the MDL was higher for BPA than those previously obtained (Weinberger et al. 2013) primarily because compromise MS conditions were required to analyze the BPXs and 4-NP, the principal focus of this study. The higher MDL for BPA with this method accounted for the lower frequency of detection for BPA, compared to other studies.

Another limitation was the relatively small sample size of 30. Future research should include a larger sample of subjects, who could be enrolled earlier in pregnancy, during a routine office visit in the last trimester. This would allow for the study to be split into two comparison groups: mothers planning for vaginal delivery and another group for Caesarean delivery. These two groups could be compared to determine whether the half-life of BPA in humans is similar for the other analogs included in this study.

Conclusions

This study successfully employed a novel analytical method for BPA and BP analogs most common in commerce today: BPB, BPE, BPF, BPS, and BPAF. A pilot sample of 30 fasting women undergoing Caesarean section was enrolled to test for detectible levels of these chemicals circulating in mothers' urine and babies' cord blood. Our findings support the pervasiveness of these chemicals in our environment and the high exposure rates among pregnant women and newborns.

Despite the relatively rapid clearance time from the body, prior research suggests the health effects of BPA and its analogs are long-lasting. Differences in metabolism and detoxification mechanisms in mothers, mechanisms facilitating or limiting transplacental transport to babies, for bisphenols should be explored in future studies. Additional research is also needed to utilize this method on a larger population, ideally in a two-armed study, to explore the presence of bisphenols in fasting and non-fasting populations. This may facilitate in determining the exact half-life of the analogs. The potential applications for this novel analysis method are extensive, suggesting it may be used on a variety of populations to further examine the presence of BPA and BP analogs in other cohorts.

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Declarations

Ethics approval and consent to participate:

To comply with best practices in ethical approval and consent, this research study was conducted in accordance with the Declaration of Helsinki and was approved prior to the commencement by the Hackensack UMC Institutional Review Board (HIRB protocol number Pro00005640).

Availability of data and materials

The de-identified clinical datasets generated and analyzed during the current study will be made available from the corresponding author, on reasonable request, to any scientist wishing to use them for non-commercial purposes.

Competing interests

The authors declare that they have no competing interests.

Standards of reporting

The STROBE Checklist was consulted during the review of this manuscript.

Disclosures

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