Parent Bisphenol A Accumulation in the Human Maternal–Fetal–Placental Unit

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Bisphenol A (BPA), an endocrine disruptor, is employed in the manufacture of a wide range of consumer products. The suggestion that BPA, at amounts to which we are exposed, alters the reproductive organs of developing rodents has caused concern. At present, no information exists concerning the exposure of human pregnant women and their fetuses to BPA. We therefore investigated blood samples from mothers (n = 37) between weeks 32 and 41 of gestation. Afer the births, we also analyzed placental tissue and umbilical cord blood from the same subjects. We developed a novel chemical derivatization–gas chromatography/mass spectrometry method to analyze parent BPA at concentrations < 1 μg/mL in plasma and tissues. Concentrations of BPA ranged from 0.3 to 18.9 ng/mL (median = 3.1 ng/mL) in maternal plasma, from 0.2 to 9.2 ng/mL (median = 2.3 ng/mL) in fetal plasma, and from 1.0 to 104.9 ng/g (median = 12.7 ng/g) in placental tissue. BPA blood concentrations were higher in male than in female fetuses. Here we demonstrate parent BPA in pregnant women and their fetuses. Exposure levels of parent BPA were found within a range typical of those used in recent animal studies and were shown to be toxic to reproductive organs of male and female offspring. We suggest that the range of BPA concentrations we measured may be related to sex differences in metabolization of parent BPA or variable maternal use of consumer products leaching BPA. *Key words:* **bisphenol A, endocrine disruptor, fetus, human, maternal blood, placenta, sex differences.** *Environ Health Perspect* **110:A703–A707 (2002). [Online 10 October 2002]**
http://ehpnet1.niehs.nih.gov/docs/2002/110pA703-A707schonfelder/abstract.html

Many different structural classes of industrial chemicals released into the environment possess potential hormonal activity or may alter normal patterns of hormone effects. Toxic effects may be produced at very low doses by interaction at hormone receptors (Triendl 2001). Many researchers hypothesize that exposure to these endocrine disruptors during critical periods of development—*in utero* or early postnatal life—could cause morphologic and functional alterations in wildlife and humans by influencing growth, reproduction, and development. Therefore, the term "endocrine disruptor" and its associated negative connotations have gained increased visibility as a public health issue. In April 2000, environment ministers of the G8 group of industrialized countries signed a communiqué stating that the risks posed by hazardous chemical substances comprised one of the greatest concerns expressed by the people of their countries (Loder 2000).

The origins of the endocrine disruptor hypothesis can be traced to reports on adolescent daughters born to pregnant women who had taken the highly potent synthetic estrogen diethylstilbestrol (DES). These girls developed a wide range of reproductive tract abnormalities including a rare form of vaginal cancer, vaginal adenocarcinoma (Herbst et al. 1971).

Bisphenol A (BPA), a chemical estrogen (Dodds and Lawson 1936), is produced in high amounts. Approximately 210,000 tons of BPA are produced in Germany per year for a wide range of applications such as flame retardants and resins (epoxy, 30%; polycarbonate, 70%) (Leisewitz and Schwarz 1998). These resins are used in dental fillings, food containers, plastic baby bottles, containers for mineral water storage, and food and beverage can linings. The ability of BPA to migrate from polymer to food, especially at high temperatures, has been described—for example, when canned food is heat processed or plastic dishes are used in the microwave (Brotons et al. 1995; Olea et al. 1996; Yamamoto and Yasuhara 1999; Yoshida et al. 2001). Leaching of BPA appears to increase with repeated use of plastic products. This may be responsible for the small amounts of BPA that are detectable in tap and river water (Khim et al. 2001; Motoyama et al. 1999; Shin et al. 2001).

In rats, it has been shown that a major percentage of orally administered ^{14}C-BPA is excreted in feces and urine. ^{14}C-BPA-monoglucuronide was determined as the major urine metabolite (Pottenger et al. 2000). Parent ^{14}C-BPA and other metabolites can also be detected, but at levels much lower than those of the BPA-monoglucuronide (< 2% of the total ^{14}C-BPA) (Pottenger et al. 2000). Parent BPA is the major component detected in feces because it may pass through the intestinal tract unchanged or the glucuronide form may be transported into the intestine via bile and become hydrolyzed (Pottenger et al. 2000). BPA-monoglucuronide is biologically inactive (Matthews et al. 2001). Rapid conversion to the monoglucuronide after oral administration of BPA results in low bioavailability of parent BPA, which is the active form. Experiments demonstrating that minuscule amounts of parent BPA alter the reproductive organs of developing mice recently sparked the greatest alarm concerning this substance. Exposure of rodent fetuses to BPA at a very low dose (below the no-observed-adverse-effect level) typical of environmental exposure was found to produce postnatal estrogenic effects: reduced daily sperm production in males, increased prostate gland weight, alteration in the development and tissue organization of the mammary gland, disruption of sexual differentiation in the brain, long-term deleterious effects in the vagina, and accelerated growth and puberty in females (Howdeshell et al. 1999; Kubo et al. 2001; Markey et al. 2001; Nagel et al. 1997; Schonfelder et al. 2002; Talsness et al. 2000; vom Saal et al. 1998; Welshons et al. 1999). Some groups, however, were unable to reproduce the effects on the reproductive tract (Ashby et al. 1999; Cagen et al. 1999a, 1999b), leading to a controversial discussion of their potentially harmful effects. The ubiquitous use of BPA has made many scientists and regulatory agencies concerned that human exposure to BPA may occur at levels shown to have adverse effects in rodent models. Until now, no data exist concerning exposure of pregnant women and their fetuses to parent BPA. A procedure with a low limit of detection is necessary to measure the levels of parent BPA that are estimated to occur in humans by typical environmental exposure based on calculations from recent pharmacokinetic studies in animals (Pottenger et al. 2000; Takahashi and Oishi 2000). Most scientists, therefore, believe that the active parent form of BPA cannot be found in human plasma, especially in pregnant women and their fetuses, because of the high first-pass metabolism to the monoglucuronide.

Given the paucity of data on BPA exposure, the widespread use of BPA, and expressed concerns among researchers and the public, we undertook this study to determine parent BPA in tissue samples and human plasma from pregnant women and their fetuses. We developed an extremely sensitive gas-chromatography/mass-spectrometry (GC/MS) method that

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This work was supported by the Benjamin Franklin Medical Center.

Received 15 April 2002; accepted 15 July 2002.

also excluded false positive results occurring from leaching of parent BPA from reaction solvents into human samples. This method will enable us to perform further epidemiologic studies on human maternal exposure to BPA and possible effects in offspring.

Materials and Methods

We undertook this study between October 2000 and May 2001 at the Benjamin Franklin Medical Center. Mothers lived in urban areas. Tissue and blood collection was performed according to the standard guidelines set by the ethical committee of the Benjamin Franklin Medical Center, Freie Universität Berlin. As women from the Berlin area arrived to give birth at the hospital, we asked them to participate in the study. All the women agreed and only those from whom we were unable to obtain human umbilical cord blood and placental tissue were omitted. Data from physical examinations, laboratory tests, and questionnaires from the Department of Gynecology and Obstetrics were recorded. Blood samples from mothers (n = 37 Caucasian females; Table 1) were collected between week 32 and 41 of gestation. Human umbilical cord blood from the same subjects was taken from the umbilical vein after the placenta was expelled. Blood (4 mL) was collected in a sterile vacutainer containing K_2EDTA (Becton Dickinson Vacutainer Systems Europe, Plymouth, UK) via a vacutainer needle (both not leaching BPA) that was directly placed into the patient's or the umbilical vein. Plasma was obtained by centrifugation of cord and maternal blood samples. Placental tissues from the same subjects were homogenised.

Plasma and tissue BPA concentrations were determined by GC/MS. The samples were analyzed in a double-blinded manner. Placental homogenate samples (0.5 g) were transferred into 10 mL glass vials and 0.5 mL H_2O and 1.0 mL of ethyl acetate (Merck, Berlin, Germany) containing the internal standard 2,2-bis-(4-hydroxy-3-methylphenyl)-propane (BPC; Aldrich, Taufkirchen, Germany) were added. Plasma samples (50 µL) were transferred into 1-mL reaction vials with 200 µL ethyl acetate containing the internal standard. The vials were sealed with Teflon-coated crimp caps and gently agitated for 30 min. Then 100 µL of the supernatant

Table 1. Characteristics of mothers and children.

Characteristics	Median (range) n = 37
Maternal age (years)	33 (22–44)
Maternal height (cm)	165 (150–182)
Maternal weight at delivery (kg)	78 (54–120)
Grava	2 (1–6)
Children	
Male	24 (65%)
Female	13 (35%)
Gestational age at delivery (weeks)	39 (32–41)
Birth weight (g)	3,490 (1,820–4,520)

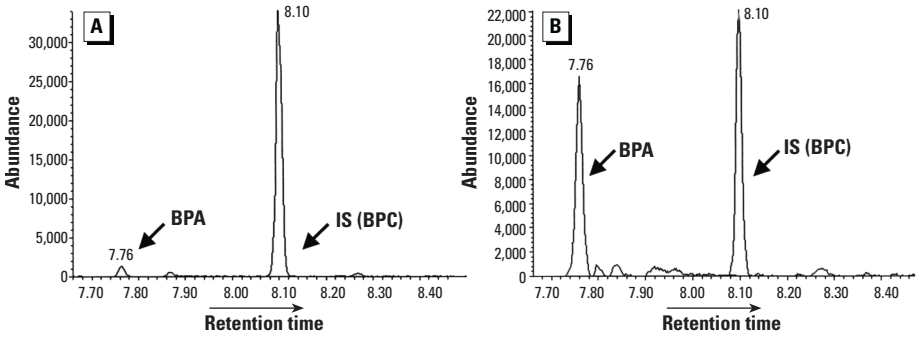

Figure 1. Comparison of parent bisphenol A contamination using either an external derivatization or an internal derivatization procedure for BPC. In the representative GC/MS chromatography, retention time for the reference (internal) standard (IS) BPC was 8.10 min and 7.76 min for the parent BPA. External derivatization of BPC (*A*) leads to less BPA contamination than does internal derivatization (*B*).

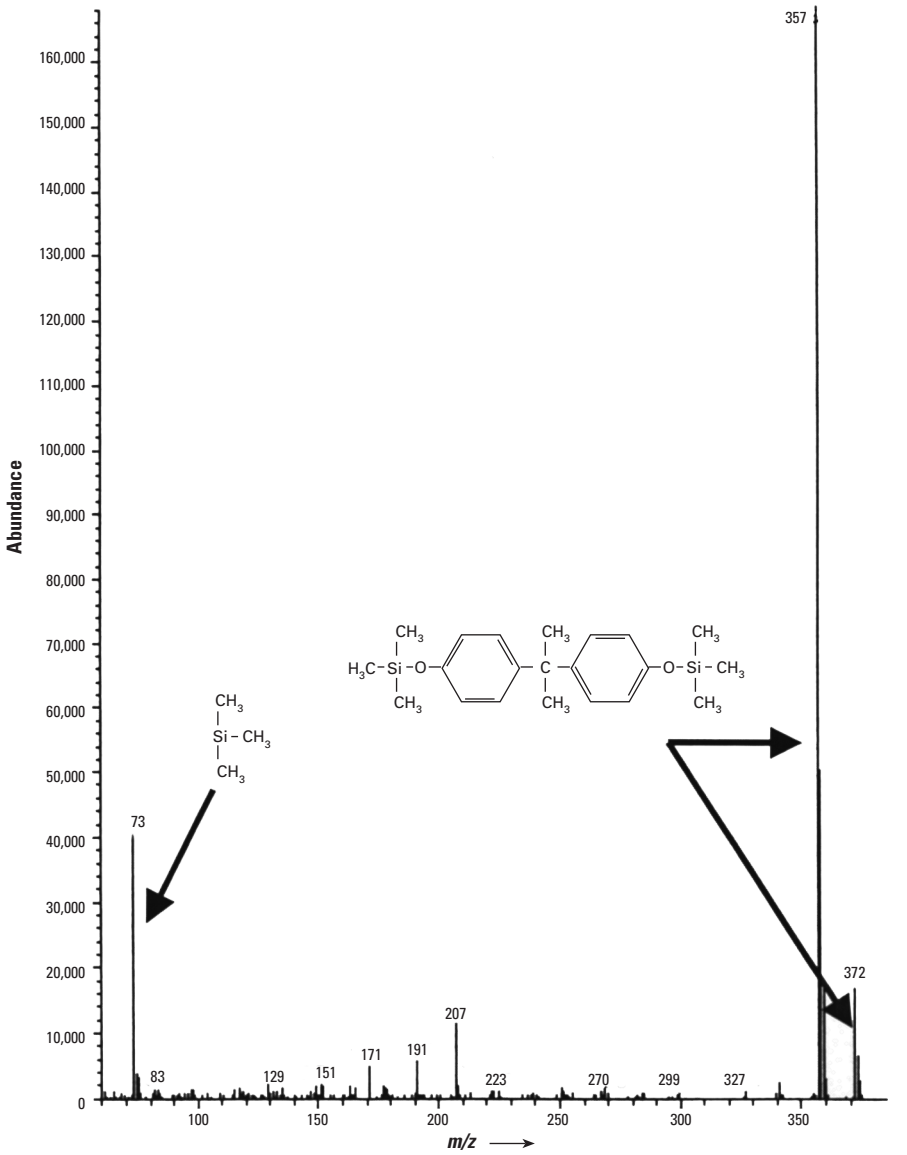

Figure 2. GC/MS spectrum to identify parent BPA in the eluted peak at 7.76 min retention time. The mass spectrum of the eluted peak at 7.76 min revealed ions at m/z 372 (M^+ion) for BPA-trimethylsilyl and at m/z 357 (M^-15 ion) for the demethylated BPA-trimethylsilyl.

were transferred to 1-mL glass reaction vials, and the extracts were concentrated under a stream of nitrogen.

To avoid BPA contamination occurring from leaching of parent BPA from the derivatization reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sulpeco, Deisenhofen, Germany), into the human samples, the vials with the dry samples were placed in 5-mL screw cap septum glass reaction vials (so-called external derivatization) containing 100 µL BSTFA, instead of directly adding BSTFA to the dry samples (internal derivatization). The vials were sealed with Teflon/silicone cap liners and heated in an oven (50°C) for 20 min. The derivatization took place without BPA contamination from the reagent. The 1-mL glass vials were removed and 50 µL ethyl acetate were added. Samples of 1 µL were injected splitless into the GC-MSD system (Hewlett Packard 5890 Series II gas chromatograph coupled to a HP 5971A mass selective detector; Hewlett Packard, Waldbronn, Germany). The GC separations were achieved with a 10 m × 0.18 mm Rtx-5 fused silica capillary column with 0.2 µm film thickness from Restek GmbH (Sulzbach, Germany) with helium as carrier gas. The initial temperature of 90°C was held for 1 min and then raised at 25°C/min to 250°C. The injector temperature was 280°C and the transfer line temperature was 250°C. The mass selective detector was operated by a HP-DOS Chemstation (Hewlett Packard) in electron impact and multi-ion detection mode using *m/z* 372 (M^+ion) for BPA-trimethylsilyl and *m/z* 385 for BPC as the internal standard. In GC/MS chromatography, retention times for the internal standard were 8.10 min and 7.76 min for the parent BPA (Figure 1). The mass spectrum of the eluted peak at 7.76 min revealed ions at *m/z* 372 (M^+ion) for BPA-trimethylsilyl and at *m/z* 357 (M^-15 ion) for the demethylated BPA-trimethylsilyl (Figure 2). Mass spectra from parent BPA and the internal standard were obtained using the same system operated in scan mode with a mass range from *m/z* 60 to *m/z* 450 with 2 scans/sec. For quantification of parent BPA, the plasma and tissue samples were prepared by spiking with BPA. The concentrations of BPA ranged from 0.1 to 200 ng/mL. The level of parent BPA in the samples was calculated based on the ratio of parent BPA to internal standard peak area response. The slope of the plot (peak-area ratio vs. amount of BPA added) indicated a linear dependence ($r = 0.9985$).

Additional control experiments were performed to check whether BPA leached from the GC/MS itself as well as storage containers and all the tubes and vessels used to obtain blood from patients. The migration test was conducted by collecting water (Lichrosolve; Merck KGaA, Darmstadt, Germany) instead of human samples in sterile vacutainers containing K_2EDTA (Becton Dickinson Vacutainer Systems Europe) via vacutainer needles. Afterwards, the GC/MS analysis was performed following the same procedures.

Data analysis was performed using the Statistical Package for Social Sciences, versions 10.0 for Windows, (SPSS, Chicago, IL, USA). Values are given as mean ± SD if not otherwise indicated. BPA concentrations in maternal plasma, fetal plasma, and placental tissue were tested for normal distribution using Kolmogorov-Smirnov-test (KS-test). Additionally, the significance of the sex difference in fetal plasma BPA levels and the sex difference in fetal plasma BPA levels related

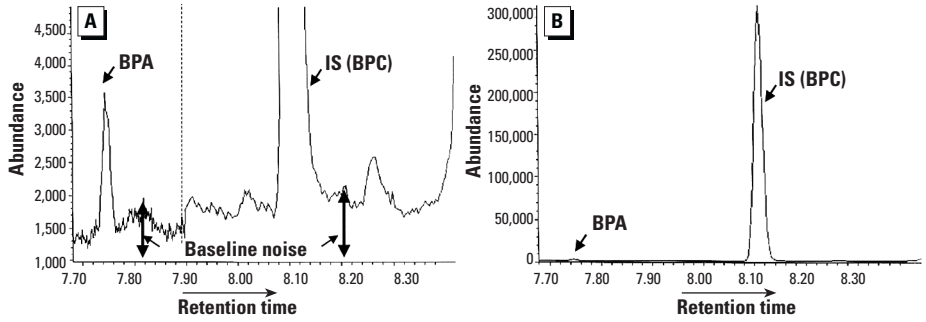

Figure 3. GC/MS spectrum to identify parent BPA. The lower detection limit (LOD = 0.010 ng/mL) of the parent substance was determined as the lowest concentration giving a response three times the average baseline noise (*A*). Additional control experiments determined that BPA was below the LOQ in all the storage containers and tubes and vessels used in obtaining blood from patients (*B*). BPC was used as internal standard.

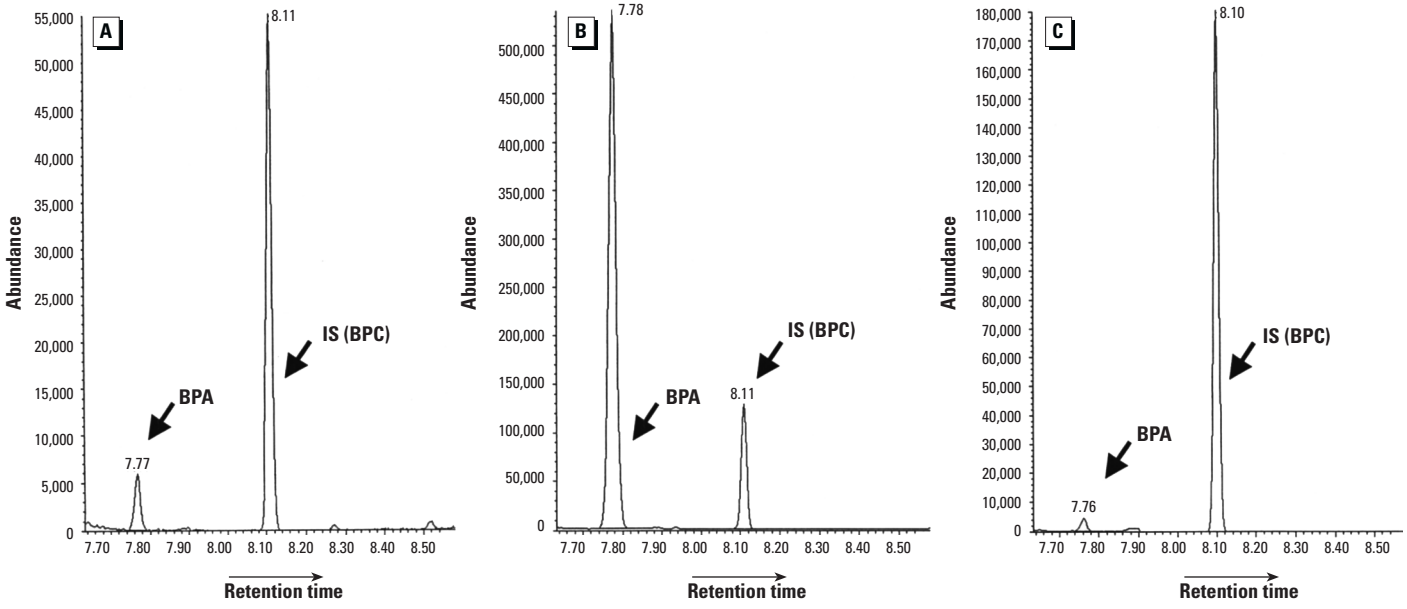

Figure 4. Ion-chromatogram to quantify parent BPA in human samples. Our GC/MS method was able to detect BPA in human samples. Representative ion-chromatogram demonstrates parent BPA in human maternal plasma (*A*), placenta (*B*), and fetal plasma (*C*) at gestational week 40 of a normal pregnancy. BPC was used as internal standard.

to birth weight (birth weight followed Gaussian distribution) were tested with the paired *t*-test. Furthermore, Fisher´s exact test was used to analyze the 24 consecutive newborn males whose fetal BPA plasma levels were higher than the maternal plasma levels, for comparison with that of 13 consecutive newborn female children.

Results

We were able to achieve a lower limit of quantification (LOQ = 0.1 ng/mL of plasma) of parent BPA using this newly developed external derivatization method (Figure 1A), compared to internal derivatization (Figure 1B). The lower detection limit (LOD = 0.010 ng/mL) of the parent substance was determined as the lowest concentration giving a response three-times the average baseline noise (Figure 3A).

Additional control experiments determined that BPA was below the LOQ in all the storage containers, tubes, and vessels used to obtain blood from patients (Figure 3B).

Our GC/MS method was able to detect BPA in human samples (Figure 4). The quantitative analyses measured parent BPA in all the human samples tested (Figure 5A). The KS-test revealed a normal distribution of parent BPA levels in maternal plasma, fetal plasma, and placental tissue. Concentrations of parent BPA (Figure 5A) in maternal plasma ranged from 0.3 ng/mL to 18.9 ng/mL (4.4 ± 3.9 ng/mL, median = 3.1 ng/mL; quartile 1 = 1.8 ng/mL; quartile 3 = 7.1 ng/mL; $n = 37$); in fetal plasma from 0.2 ng/mL to 9.2 ng/mL (2.9 ± 2.5 ng/mL, median = 2.3 ng/mL; quartile 1 = 1.1 ng/mL; quartile 3 = 5.2 ng/mL; $n = 37$); and in placental tissue from 1.0 ng/g to 104.9 ng/g (11.2 ± 9.1 ng/g, median = 12.7 ng/g; quartile 1 = 3.6 ng/g; quartile 3 = 22.5 ng/g; $n = 37$).

In some cases, fetal plasma levels of parent BPA were higher than those for maternal blood ($n = 14$; Figure 5B; Table 2). When we considered just these cases, the percentage of cases with higher levels of parent BPA in fetal plasma was significantly higher in the male group (12 of 24 cases) than in the female group (2 of 13 cases) ($p = 0.0402$, Fisher´s exact test).

In addition, the paired *t*-test revealed significantly higher levels ($p = 0.016$) of BPA in male fetal plasma (Figure 6A; 3.5 ± 2.7 ng/mL, median = 2.8 ng/mL, quartile 1 = 1.15 ng/mL; quartile 3 = 5.35 ng/mL) than in female (1.7 ± 1.5 ng/mL, median = 1.3; quartile 1 = 0.5 ng/mL; quartile 3 = 2.4 ng/mL). When fetal BPA concentrations were correlated to birth weight (bw), the paired *t*-test revealed a significantly higher level ($p = 0.012$) of BPA in male fetal plasma [1.0 ± 0.8 (ng BPA/mL)/kg bw; median = 0.8 (ng BPA/mL)/kg bw than in female (0.5 ± 0.4 (ng BPA/mL)/kg bw; median = 0.5 (ng BPA/mL)/kg bw] (Figure 6B).

Conclusions

This is the first study demonstrating parent BPA in human samples of pregnant women and their fetuses.

The issue of bioavailability of parent BPA in humans, especially in pregnant women and their fetuses, has been contentious. It has been generally suggested that after oral administration, parent BPA is partially absorbed and rapidly excreted (half-life is less than 1 day) with no evidence of bioaccumulation in tissues. It was therefore uncertain whether parent BPA circulated at concentrations shown to be toxic to reproductive organs of male and female offspring of mice and rats (Nagel et al. 1997). Our quantitative analyses detected parent BPA from 0.3 ng/mL to 18.9 ng/mL in maternal plasma, and in fetal plasma in the range between 0.2 ng/mL to 9.2 ng/mL. Data from a recent study reported by Takeuchi and Tsutsumi (2002) support our findings that parent BPA accumulation can be measured in humans and that there might be sex differences. Unlike these findings, we could detect BPA at a broader range in serum, not just at concentrations around 1 ng/mL. In contrast to our study and the reports of Inoue et al. (2001) and Ohkuma H. et al. (2002), Fung et al. (2000) did not detect BPA in serum of 40 healthy subjects when investigating the pharmacokinetics of BPA released from a dental sealant.

Here, we show that humans have levels of parent BPA in the range of those used in recent animal studies (Pottenger et al. 2000), where differences in the concentration–time profiles of parent BPA, based on dose and sex, could be demonstrated. Parent BPA could not be quantified after oral administration of 10 mg BPA/kg bw to male rats, but was quantifiable for over 22 hr after oral administration of this dose to female rats (C_{max} = 40 ng BPA/g). Oral administration of 100 mg BPA/kg bw resulted in about a 10-fold increase in the concentration of parent BPA (C_{max} = 2.290 ng/g) in the blood of female rats, compared with the 10-mg dose, and was quantifiable in blood through the last sample analyzed at 12 hr after administration. In contrast, concentrations of parent BPA in the blood of males dosed with 100 mg BPA/kg BW were about 10 times less (C_{max} = 220 ng/g). Furthermore, previous studies demonstrated that sex and

Table 2. Maternal and fetal plasma levels of parent BPA (ng/mL).

Sex (fetus)	Gestational week	Maternal plasma	Fetal plasma
Male	40	4.3	9.2
Male	40	3.5	6.8
Male	40	1.9	5.2
Male	39	2.0	3.7
Male	39	4.5	4.7
Male	39	1.7	2.5
Male	39	2.5	3.6
Male	38	0.3	1.1
Male	38	4.2	5.4
Male	37	0.9	1.8
Male	36	1.8	6.5
Male	35	1.1	7.7
Female	39	2.5	2.9
Female	38	1.1	2.9

In some cases ($n = 14$), fetal plasma levels of parent BPA (ng/mL) were higher than that for maternal blood.

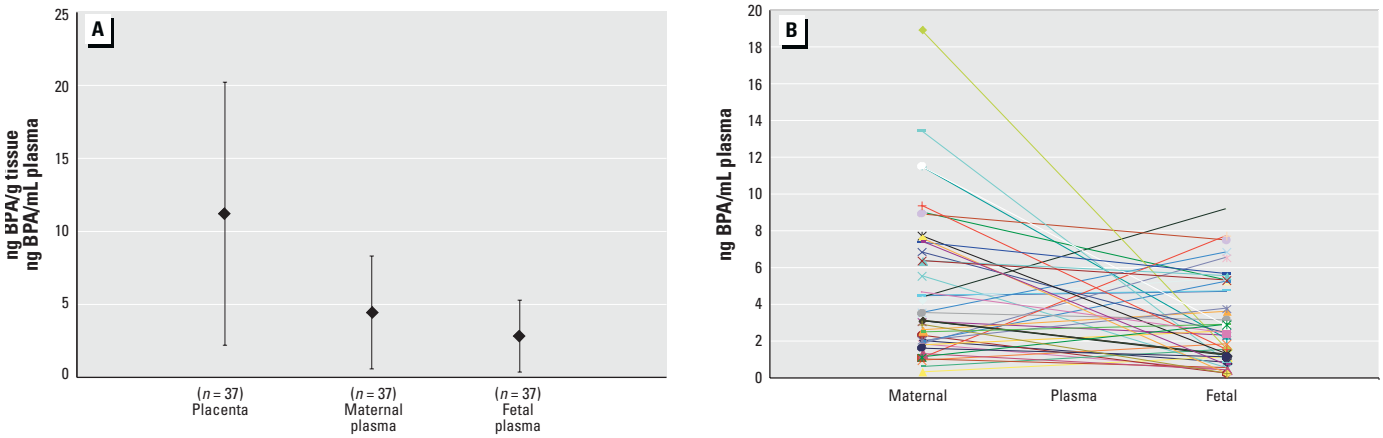

Figure 5. Parent bisphenol A levels in placental tissue and maternal and fetal plasma from pregnancies ($n = 37$). (*A*) The KS-test revealed a normal distribution of parent BPA levels in maternal plasma, fetal plasma, and placental tissue. (*B*) In some cases, fetal plasma levels of parent BPA were higher than those for maternal blood ($n = 14$).

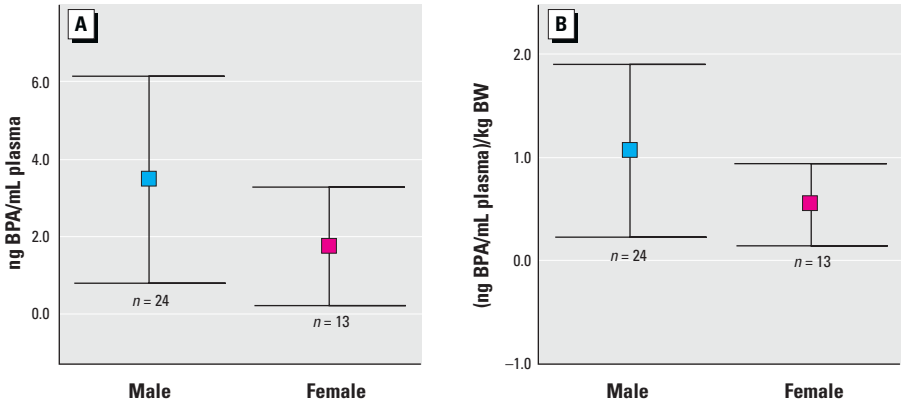

Figure 6. Sex differences in BPA concentrations of fetal plasma (*A*) and after fetal BPA concentrations were correlated bo body weight (*B*). The paired *t*-test revealed an increase ($p = 0.016$) in BPA fetal plasma concentrations (*A*) in males. When fetal BPA concentrations were correlated to body weight (*B*), the paired *t*-test revealed a significant increase ($p = 0.012$) in fetal plasma concentrations in males compared to females. Error bars indicate SD.

long-term soy diets affect the metabolism and excretion of phytoestrogens. The excretion half-life shortened progressively in women but lengthened progressively in men (Lu and Anderson 1998). Thus, we suggest that the range of BPA concentrations we measured may be related to sex differences in metabolization of free BPA or variable maternal use of consumer products leaching BPA. In accordance with a recent rat study (Takahashi and Oishi 2000), our findings demonstrate that the human placenta does not act as a barrier to parent BPA. The rate of clearance of BPA is slower in the fetus than in maternal blood (Takahashi and Oishi 2000), because most uridinediphosphate–glucoronosyltransferase isoenzymes are not expressed until after birth, with the full complement being expressed by 3 months of age (Coughtrie et al. 1988).

The etiology of many adverse reproductive outcomes among humans is poorly understood. A growing body of scientific evidence indicates that a number of chemicals to which humans are in contact—including natural and synthetic hormones, organometals, pesticides, persistent environmental pollutants, monomers, and additives used in the plastic industry—may interfere with the endocrine system, potentially causing adverse effects to both wildlife and humans. Reasons for concern include evidence for a number of trends: There are indications for an increase in the incidence of some hormonally sensitive carcinomas, decrease in sperm count and quality, and increased obesity and earlier puberty occurring in girls, as well as altered physical and mental development in children. To date, there is no evidence that ingestion of BPA at levels estimated to occur by typical environmental exposure has adverse effects in humans; a causal relationship of the observed effects with BPA has not yet been adequately established. Long-term follow-up studies are needed to assess the adverse effects of BPA exposure in early life. Further studies on human exposure to BPA are needed to address the question whether maternal exposure to BPA can lead to adverse health effects in offspring. Our method allows us to investigate parent BPA levels in small human sample volumes to perform those exposure studies on the human population.

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