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A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry

Ivana Kosarac[a], Cariton Kubwabo[a,*], Kaela Lalonde[a], Warren Foster[b]

[a] Environmental and Radiation Health Sciences Directorate, Health Canada, Ottawa, ON, Canada K1A 0K9
[b] McMaster University, Department of Obstetrics and Gynecology, Hamilton, ON, Canada L8N 3Z5

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ABSTRACT

Bisphenol A is widely used as a monomer in the manufacture of polycarbonates and epoxy resins, as an antioxidant in polyvinyl chloride (PVC) plastics and as an inhibitor of end polymerisation in PVC. Several different methods have been used to quantify total BPA in biological specimens. However, quantification of both free and conjugated BPA continues to present challenges. Moreover, there is limited data concerning fetal exposure. Therefore, the objective of this study was to develop a new method for the analysis of both free and conjugated BPA in human maternal and umbilical cord blood serum. For the analysis of free BPA, the method consisted of a liquid–liquid extraction followed by a two-step solid-phase extraction sample cleanup on Florisil and Oasis HLB sorbents, derivatization of the extract using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and analysis by gas chromatography/tandem mass spectrometry (GC/EI-MS/MS). To determine the amount of conjugated BPA in serum samples, bisphenol A-d6 β-glucuronide (4-[1-(4-hydroxyphenyl)-1-methylethyl-d6]phenyl β-D-glucopyranosiduronic acid) was added to each sample prior to enzymatic deconjugation. The MDL and LOQ for BPA were 0.026 ng/mL and 0.087 ng/mL, respectively. The observed recoveries ranged between 65% and 88%. The new method was applied to the determination of paired human maternal and umbilical cord blood serum samples. The results demonstrated that total BPA concentrations in human maternal serum at mid-pregnancy and at delivery ranged from <0.026 ng/mL to 10.425 ng/mL (median 0.548 ng/mL, $n = 12$) and <0.026 ng/mL to 3.048 ng/mL (median 1.461 ng/mL), respectively. Results for matching umbilical cord blood serum BPA concentrations were in the range of <0.026–2.569 ng/mL (median 1.823 ng/mL). The concentrations measured in this study agreed well with BPA levels in human serum reported internationally. Only 2 mid-pregnancy serum samples out of 12 contained quantifiable amounts of conjugated BPA, indicating that BPA–glucuronide is not abundant in either human maternal or umbilical cord blood serum.

1. Introduction

The first documented synthesis of bisphenol A (BPA) was reported in 1905 by Thomas Zincke of the University of Marburg, Germany, who described its synthesis from phenol and acetone [1]. In spite of this chemical being available for a long time, it was not until 1953 that Schnell and Fox developed manufacturing techniques for utilizing BPA as a building monomer for a lightweight and versatile polycarbonate [2]. Owing to its unique physical properties, relative ease and cost-effectiveness of manufacturing, polycarbonate and other BPA-based industrial materials have been used extensively in many consumer products. Polycarbonates are widely used in food contact plastics such as reusable beverage bottles, infant feeding bottles, tableware and storage containers, whereas epoxy resins are used in protective linings for food and beverage cans [3]. BPA exposure has been linked with a number of adverse health outcomes in several studies which have been summarized elsewhere [3,4].

Despite the continuing debate regarding the interpretation of scientific data on the potential toxic effects of BPA, it is generally agreed that newborn and young children are the most at risk since their bodies are in the early stages of development [5]. BPA has been well monitored in environmental samples through which humans can be potentially exposed to such as drinking water, food, and food contact materials. However, there have only been a few studies on the biomonitoring of this contaminant in humans and a better understanding of BPA occurrence and distribution in the human population is needed. The measurement of unconjugated

* Corresponding author. Tel.: +1 613 941 6081; fax: +1 613 946 3573.
E-mail address: Cariton.Kubwabo@hc-sc.gc.ca (C. Kubwabo).

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(free) BPA and its glucuronidated and sulphated conjugates, in a number of biological tissues and fluids, will allow for an estimation of BPA concentrations in humans. Only BPA in its free form is shown to have estrogenic activity [6], however, BPA–glucuronide may cross the placenta and affect the fetus by reactivation to free BPA; this was demonstrated in humans and rats [7,8]. The evaluation of "*in utero* exposure" to bisphenol A, through the analysis of biological fluids from pregnant or nursing mother (i.e. blood, urine, breast milk, colostrum), the fetus or newborn infant (i.e. meconium, umbilical cord blood, neonatal urine), and from both the fetus and the mother (i.e. placental tissues, amniotic fluid), would allow for a better understanding and a more concrete picture into the exposure of the most vulnerable segment of the human population. The identification and quantification of BPA are challenging due to the low concentrations at which the compound is typically present in biological matrices. Thus improved methods are needed to better characterize human exposure to this ubiquitous contaminant. For biological samples, cleanup and analytical methods for BPA determination are complex. One form of biomonitoring of BPA is measuring its levels in human serum. Three main types of sample preparation for the analysis of BPA in serum have been employed and include on-line extraction [9,10], liquid–liquid extraction [11], solid phase extraction [12–15] or no extraction at all [16].

The analysis of BPA in human and/or umbilical cord blood serum has involved chromatographic separation (either gas or liquid) coupled to an array of detection techniques. Liquid chromatography (LC) has been used with fluorescence detection [17–19], electrochemical detection [20] and mass spectrometry [9,11,21]. Gas chromatography–mass spectrometry (GC/MS) has been used with different ionization modes, including negative ion chemical ionization [22], electron capture negative ionization [12,13] and electron impact ionization in single ion monitoring mode [14,23]. In addition, methods have been developed using immunochemical techniques for the determination of BPA in serum. The most commonly used immunochemical technique for the determination of BPA in serum is the Enzyme Linked Immunoabsorbent Assay (ELISA) method [15,16,24,25]. Although immunochemical techniques are thought to be more cost-effective, simpler and have a higher throughput than chromatography-related analytical techniques such as GC and LC [16], these studies have been criticized due to the possibility of interference from other compounds and poor sensitivity [26]. Moreover, due to the complexity of the human serum matrix and very low concentrations of free BPA in serum, methods that provide ultra-low detection limits need to be developed. While studies have measured free BPA concentrations in serum samples, there continues to be a shortage of studies measuring the levels of both free BPA and its conjugated form, BPA–glucuronide, in human maternal serum and their matching umbilical cord blood serum samples.

The scarcity of human serum BPA data and lack of reliable analytical methods have been the driving force behind the development of a specific and sensitive method for quantitative determination of both free and conjugated bisphenol A in human maternal and umbilical cord blood serum, using a mass labelled bisphenol A-d6 β-glucuronide in addition to a $^{13}C_{12}$-BPA internal standard. Sample cleanup was achieved via solid phase extraction; derivatization was performed using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and the analysis by GC/EI-MS/MS.

2. Materials and methods

2.1. Serum samples

Human maternal and umbilical cord blood serum samples were collected from pregnant women between 2004 and 2005 as part of the FAMILY (Family Atherosclerosis Monitoring In Early Life) study conducted at McMaster University (Ontario, Canada). Blood was collected from an anticubital vein using a 19 gauge needle and vacutainer with a 10 mL serum separator tube and allowed to clot at 4 °C for 4 h. Samples were centrifuged for 20 min at 3000 rpm, the serum decanted, and serum samples were stored at −20 °C until analysis. Matched human maternal at mid-pregnancy, at delivery and umbilical cord blood serum samples from 12 pregnant women were obtained for this study. The determination of emerging persistent organic pollutants in archived samples from the FAMILY study samples was approved by McMaster University and Health Canada's Research Ethics Boards.

2.2. Standards and reagents

BPA standard (99%) was purchased from AccuStandard (New Haven, CT, USA). $^{13}C_{12}$-BPA standard was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Bisphenol A-d6 β-D-glucuronide (4-[1-(4-hydroxyphenyl)-1-methylethyl-d6]phenyl β-D-glucopyranosiduronic acid) and bisphenol A-d6 (2,2-bis(4-hydroxyphenyl)propane-*methyl*-d6) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Reagent grade ethanol was obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Methanol, dichloromethane, GC grade hexane and acetone, and HPLC-grade water were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 98.1%) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA). Ammonium sulphate (reagent plus) and 778,000 units/g solid β-glucuronidase from *Escherichia coli* (Type X-A) were products of Sigma–Aldrich (St. Louis, MO, USA). The β-glucuronidase enzyme was made into a working enzyme solution of 3.213 g/μL with HPLC-grade water.

2.3. Deconjugation of BPA glucuronide

Each 1 mL of serum sample was fortified with 40 μL of a 500 ng/mL bisphenol A-d6 β-D-glucuronide spiking solution and vortexed. Following the mixing, 50 μL of β-glucuronidase from *E. coli* source were added, and the samples were then incubated for 45 min at 37 °C.

2.4. Liquid–liquid extraction

Each serum sample was split into two 1 mL aliquots: one for deconjugation with enzyme and incubation, and one was an untreated duplicate. Following the incubation of the enzyme-treated sample, both aliquots were fortified with 20 μL of 500 ng/mL $^{13}C_{12}$-BPA spiking solution. 1 mL of saturated aqueous ammonium sulphate, 2 mL of ethanol and 4 mL of hexane were added to each sample. The samples were gently inverted a few times and finally left to settle for a few minutes to allow for a clear phase separation. The top organic layer, which contained non-polar fats was discarded and 4 mL of hexane were added to the bottom phase to remove any residual non-polar fats and related compounds. After invert mixing and removal of the top hexane layer, 3 mL of dichloromethane were added to the remaining bottom layer. The samples were then inverted, mixed and sonicated for 10 min in order to allow for a clear phase separation. The bottom dichloromethane layer was carefully transferred with pre-cleaned Pasteur pipette into a clean 15 mL solvent cleaned glass centrifuge tube. The solvent was removed by evaporation to dryness under a gentle stream of nitrogen at 30 °C, and the sample was reconstituted in 2 mL of HPLC-grade water.

2.5. Sample cleanup

Sample cleanup steps consisted of two solid phase extractions. In the first step, 2 mL reconstituted extracts in HPLC-grade water were loaded onto a preconditioned (dichloromethane, hexane) 1 g Envi-Florisil glass cartridge (Supelco, Bellefonte, PA, USA). First, cartridges were washed with 10 mL of hexane, and this fraction was discarded as it contained residual fats. Next, the analyte was eluted with 25 mL of dichloromethane into a solvent pre-rinsed 40 mL glass vial. Using a gentle stream of nitrogen, samples were evaporated to dryness and then reconstituted in 3 mL of HPLC-grade water. In the second step of the solid phase clean up, extracts were loaded onto a preconditioned (9 mL acetone, 9 mL methanol and 5 mL of HPLC-grade water) 200 mg Oasis HLB glass cartridge (Waters, CA, USA). SPE cartridges were allowed to dry for 30 min prior to solvent elution. The analyte was eluted using 6 mL of methanol and 5 mL of acetone in succession. The extracts were concentrated to dryness under a gentle stream of nitrogen and reconstituted in 150 μL of acetone. 50 μL of MSTFA were added to each sample for derivatization prior to GC/EI-MS/MS analysis.

2.6. GC/EI-MS/MS analysis

The chromatographic separation was performed on a Zebron ZB-5HT capillary column (30 m × 0.25 mm × 0.1 μm) from Phenomenex (Torrance, CA, USA) using an Agilent 6980 gas chromatograph equipped with an Agilent 7683B Series Autosampler. A 1 μL sample was injected in splitless mode at 250 °C. The GC oven temperature programme was as follows: 60 °C (hold 1 min) to 280 °C at 10 °C/min at a flow rate of 1 mL/min in constant flow mode. Mass spectrometric experiments were performed using a Waters Quattro micro GC triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The source and GC interface temperatures were set at 180 °C and 250 °C, respectively. The MS/MS was operated in electron impact at 70 eV in multiple reaction mode (MRM). MassLynx version 4.1 was used for data acquisition and processing. The following MRM transitions (m/z) were monitored: BPA (m/z 357→191), $^{13}C_{12}$-BPA (m/z 369→197) and d6-BPA (m/z 360→73).

3. Results and discussion

3.1. Blank contamination challenge

Solvents, water, extraction equipment and method blanks were checked for the presence of target analyte prior to analysis. During the method development of previous work on the migration of bisphenol A from plastic bottles [27], the importance of minimizing background contributions during sample extraction, storage and analysis became evident because BPA is a ubiquitous chemical. A number of trials and checks were carried out in order to minimize the background levels and to find reagents and labware with the lowest possible BPA levels. Only chemically pre-cleaned glass vials, containers and pipettes were used for the analysis. Early in the method development stage, reagent water blank BPA levels were shown to vary dramatically. In one instance, laboratory reverse osmosis water was found to contain BPA levels of 6.9 ng/mL. Therefore, only very low BPA-containing HPLC-grade water was used in all of the steps of sample preparation. Moreover, plastic solid phase extraction cartridges were found to contain significantly higher levels of BPA than those packaged in glass. Blank levels ranged between <0.026 and 0.083 ng/mL. All reported results were corrected for their respective blank samples. As can be seen from Figs. 1 and 2, extensive sample cleanup, derivatization and GC/MS/MS detection provided clean mass chromatograms for BPA in serum samples.

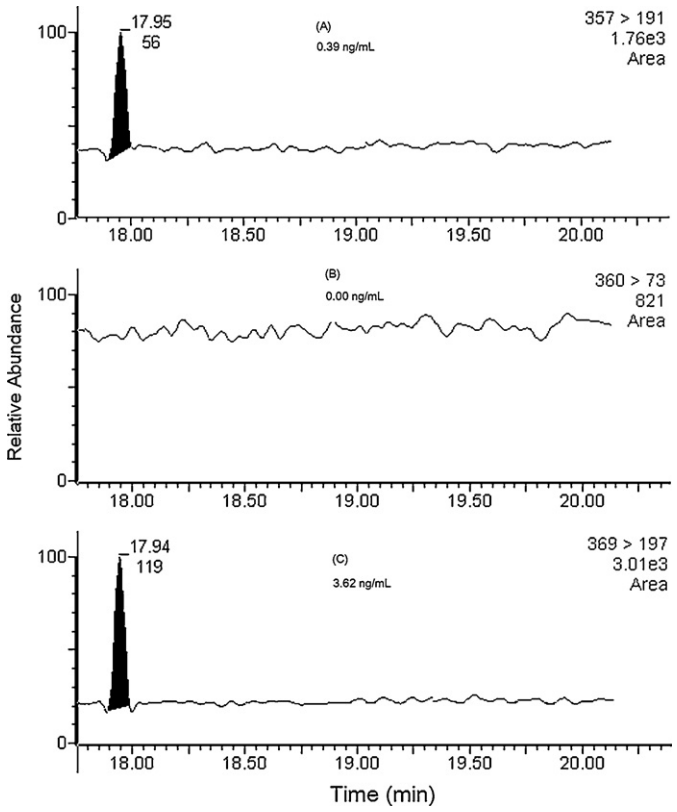

Fig. 1. MRM chromatograms of (A) free BPA, (B) d6-BPA, and (C) $^{13}C_{12}$-labelled BPA of a serum sample not enzymatically deconjugated.

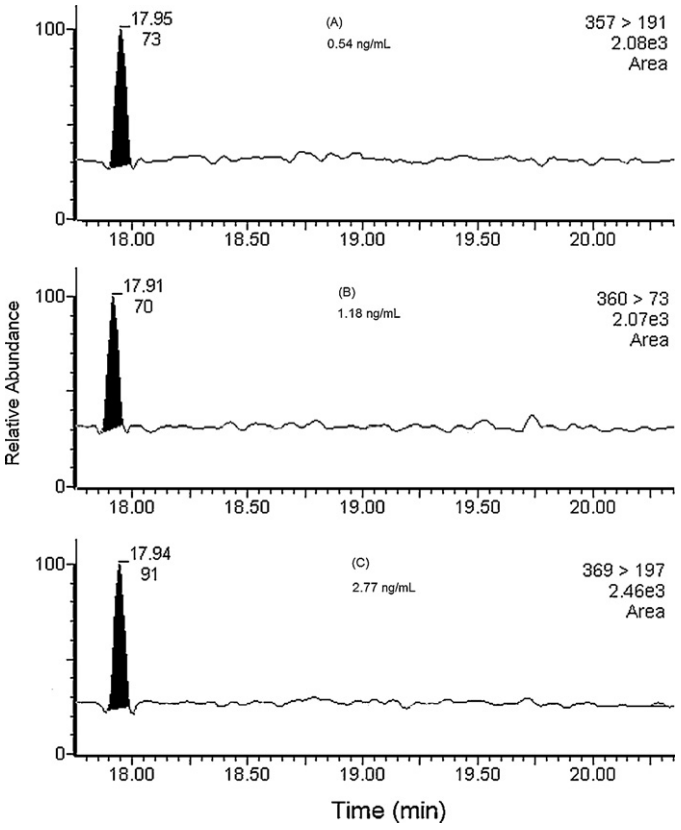

Fig. 2. MRM chromatograms of (A) free BPA, (B) d6-BPA, and (C) $^{13}C_{12}$-labelled BPA of a serum sample enzymatically deconjugated with β-glucuronidase.

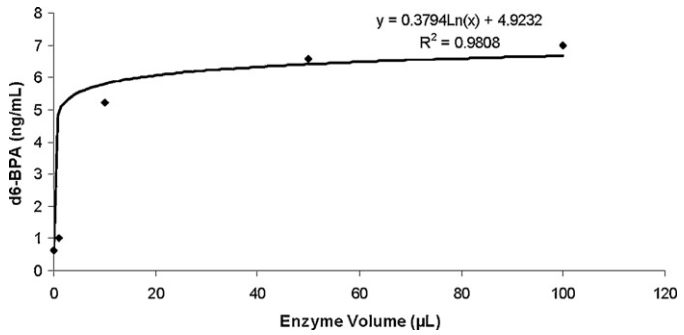

Fig. 3. d6-BPA concentration (ng/mL) relative to the volume of deconjugation enzyme (μL) added.

3.2. Method performance

The calibration curve was linear over a concentration range from 1 ng/mL to 100 ng/mL with a coefficient of correlation (r^2) greater than 0.998. The method detection limit (MDL) was determined according to the EPA Regulation 40 CFR part 136 (Appendix B) method, Revision 1.11 [28], whereby the standard deviation associated with eight replicate analyses of sheep serum samples spiked with $^{13}C_{12}$-BPA at 1 ng/mL and processed through the entire analytical procedure was multiplied by the Student's t value of 2.998 (appropriate for a 99% confidence level). The MDL for BPA was calculated to be 0.026 ng/mL. The limit of quantification (LOQ) was calculated according to the US EPA method, where the standard deviation associated with the eight replicate analyses of BPA conducted to obtain the MDL was multiplied by a factor of 10. The LOQ for BPA was 0.087 ng/mL. The recoveries for this method, estimated by spiking a known amount of carbon-13-labelled-BPA into sheep serum samples, varied between 65% and 88%. Intra-day variability of eight replicates of sheep serum was calculated at 6.2%. Inter-day reproducibility was investigated with replicates of the sheep serum sample over the course of the analysis and relative coefficient of variability was found to be 17.6%.

3.3. BPA–glucuronide

The presence of conjugated BPA–glucuronide in urine has been well documented in a number of previous studies [29–33]. However, very few studies to date have determined the levels of BPA–glucuronide in serum or plasma samples [10,32,34]. In part, the lack of data exists because the deconjugation reaction involving the enzymatic hydrolysis of glucuronide and release of free BPA is difficult to assess. The procedures used to determine the completeness of the reaction and the efficiency of the enzymatic species are often complicated, time and resource-consuming approaches. As a result, the majority of studies reported only free BPA concentration in serum. To the best of the authors' knowledge, this study is the first to employ the bisphenol A-d6 β-D-glucuronide. The use of the labelled-conjugated BPA allowed the optimization of the enzymatic reaction and permitted accurate determination of the conjugated BPA concentration in serum samples. The optimum amount of enzyme was determined using archived human serum sample fortified at 100 ng/mL with bisphenol A-d6 β-D-glucuronide. The volume of enzyme added (Fig. 3), the temperature and the time of reaction were determined during these preliminary optimization trials. The resulting deconjugation reaction's optimum conditions were determined to be: temperature at 37 °C, reaction time at 45 min and 1,292,258 kU of enzyme added. Fig. 1 shows mass chromatograms for MRM transitions of BPA (m/z 357→191), d6-BPA (m/z 360→73) and $^{13}C_{12}$-BPA (m/z 369→197) in a human serum sample that was fortified with bisphenol A-d6 β-D-glucuronide but had no addition of glucuronidase enzyme, and corresponding to a free BPA concentration of 0.39 ng/mL. As expected, d6-BPA was not detected, while Fig. 2 shows mass chromatograms for BPA in the same sample, fortified with d6-BPA β-D-glucuronide and enzymatically deconjugated with β-glucuronidase, to give total BPA and d6-BPA concentrations of 0.54 ng/mL and 1.18 ng/mL, respectively.

In spite of the deconjugation reaction under kinetic control, this study revealed that only 2 out of 12 serum samples at mid-pregnancy contained quantifiable levels of glucuronide conjugated BPA in the serum, with a concentration of 0.12 ng/mL and 0.22 ng/mL, respectively. In a recent study, Ye et al. [34] tested 15 serum samples and found that only 1 sample had a quantifiable concentration of free BPA (1.5 ng/mL), which was equivalent to the total BPA level, suggesting that this particular sample contained no BPA–glucuronide. In another study, the same research group did not detect any free or conjugated form of BPA in 16 serum samples; the poor detection frequency could be attributed to the relatively higher LOD of 0.3 ng/mL [10]. In a study conducted on 19 healthy human subjects, no free or conjugated BPA was detected in plasma samples at a concentration above the LOD of 0.5 ng/mL [32].

In the future, it would be prudent to study the mechanism and characteristics of the glucuronidase deconjugation reactions in greater detail, as well as the stability of the bisphenol A-d6 β-D-glucuronide conjugated species. Further investigation would be required to revisit the hydrolysis, the enzymatic deconjugation and the effects of adding varying amounts of labelled bisphenol A-d6 β-D-glucuronide, and to assess enzymes from different sources.

3.4. Free and conjugated BPA

This new method was applied to a pilot study of 12 individual human maternal serum samples at mid-pregnancy, at delivery and their matching umbilical cord blood serum samples. BPA derivatization with MSTFA and analysis by GC/EI-MS/MS allowed the detection and the quantification of BPA at trace levels and made possible a relatively high detection frequency of BPA in the samples. In this study, BPA concentrations in maternal serum samples at mid-pregnancy ranged from not detected to 10.4 ng/mL (median 0.548 ng/mL, detection frequency 67%); in maternal serum at delivery from not detected to 3.05 ng/mL (median 1.46 ng/mL, detection frequency 58%); and in corresponding umbilical cord blood serum samples from not detected to 2.57 ng/mL (median 1.82 ng/mL, detection frequency 42%). Although the results from this study showed that median values at mid-pregnancy were lower than those at delivery and in umbilical cord blood serum, the small sample size precluded any comparison of paired data from maternal and umbilical cord blood serum samples. However, individual results in maternal–fetal pairs in this study did not always portray this pattern (Fig. 4). In a study conducted to assess the BPA accumulation in the human maternal–fetal–placental unit (n = 37), the median concentration of BPA in maternal and in fetal plasma was 3.1 ng/mL and 2.3 ng/mL, respectively [23]. In that small study, 62% of fetal plasma samples had lower concentrations than corresponding maternal plasma. In another study published by Lee et al. [17] that examined maternal and fetal exposure to BPA, of 300 pregnant women in Korea, 84% of the maternal and 40% of the umbilical cord blood serum samples contained detectable levels of BPA, the mean concentration of total BPA in the maternal and in the umbilical cord serum samples was 9.04 (±14.03) ng/mL, and 1.13 (±1.43) ng/mL, respectively. In a more recent study [11], the mean concentration of BPA in maternal serum (n = 26) and in fetal serum (n = 25) was 0.7 (±0.1) ng/mL and less than the LOD of 0.6 ng/mL, respectively. Individual trends might in fact be more difficult to predict given the uncertainty of how much BPA each mother may be exposed to during delivery. For example, intravenous administration of a drug or a treatment during the delivery may actually have

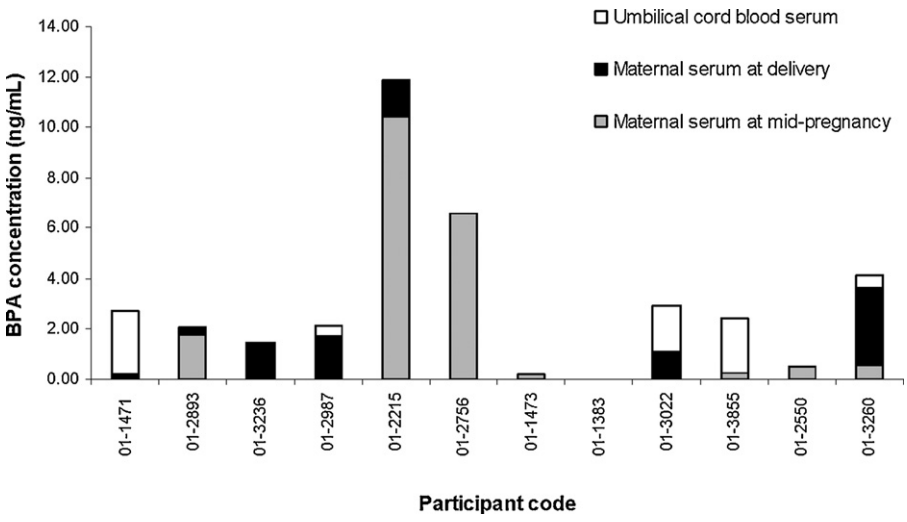

Fig. 4. Individual bisphenol A serum results at mid-pregnancy, at delivery, and in corresponding umbilical cord serum.

significant effects on the concentration of BPA due to the leaching of BPA from the medical apparatus used to deliver the same treatment. The new bisphenol A-d6 β-D-glucuronide-based method is currently being applied in a more comprehensive study designed to examine the association between developmental exposure to BPA and sex dependent developmental landmarks.

4. Conclusions

A sensitive method for the quantification of free and conjugated BPA in human maternal and umbilical cord blood serum was developed in the course of this study. To the authors' knowledge, this is the first time that deuterium labelled bisphenol A-d6 β-D-glucuronide substrate was used to quantify and control the glucuronidase deconjugation and hydrolysis reaction. BPA concentrations were determined in human maternal and umbilical cord blood serum samples of 12 maternal–fetal pairs and the results are consistent with the levels observed internationally.

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