Alkylphenols and bisphenol-A and its chlorinated derivatives in adipose tissue of children

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Abstract

Bisphenol-A (BPA) is a plastic monomer and plasticizer used in the production of polycarbonate and epoxy resins. Alkylphenols are used as antioxidants and in the synthesis of industrial detergents, in toiletries, and as spermicides. Because of their endocrine disrupting properties, there is strong interest in studying their contribution to human exposure, especially in children. Octyl (OP) and nonylphenol (NP), bisphenol-A (BPA) and its chlorinated derivatives (Cl(x)BPA) were investigated in adipose tissue of 86 children in Spain. OP and NP were above the limit of detection (LOD) in 21 and 31 out of 86 samples, respectively. Means (SD) of OP and NP were 6.27 (6.63) and 38.27 (56.91) ng/g of adipose tissue, respectively. BPA was above LOD in 53 out of 86 samples (62%). Among Cl(x)BPA, Cl2-BPA was the most frequent (99%) and abundant, followed by Cl-BPA (64%) and Cl4-BPA (12%). Means (SD) of BPA, Cl-BPA, Cl2-BPA, Cl3-BPA and Cl4-BPA were 17.46 (14.82), 7.32 (4.26), 14.45 (9.79), 4.14 (1.35) and 3.54 (1.94) ng/g, respectively. There are no published data on alkylphenols and BPA in human adipose tissue or on Cl(x)BPA in adipose tissue or blood, limiting comparisons. However, these levels are higher than BPA concentrations reported to stimulate molecular endpoints in vitro and are at levels observed to induce effects in animal models. Further research is needed to explore their combined effects on children health, and to follow trends in human exposure if uses of these chemicals are to be regulated.

Keywords: bisphenols, alkyphenols, children exposure.

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1 Introduction

BPA, a high-production volume manmade chemical, is a substrate for the production of polycarbonate resins (71%) and epoxy resins (27%). BPA is also used as an intermediate for binding, plasticizing, or hardening of plastics, paints/lacquers, binding materials, and filling-in materials Furthermore, BPA is used as an additive for flame-retardants, brake fluids, and thermal papers. The release of BPA into the environment and its use in a variety of high-volume consumer products means that there are many possible routes of exposure for individuals in the general population [1, 2] and makes plastic-to-water migrations a significant source of human exposure via drinking water. BPA in tap water may react with residual chlorine giving chlorinated derivatives as by-products of the reaction between BPA and free chlorine [3, 4].

Despite the high levels of its production, environmental release and utilization, possibly higher than that of any other single man-made chemical, BPA has not been subjected to any environmental legislative control. Non-food products, as in the case of dental composites and sealants [5,6] are significant sources of BPA exposure, but the importance of environmental BPA exposure for the general population has yet to be established [7]. Food is acknowledged to be the main source of exposure to bisphenols [8], in part due to BPA migration from food containers. Epoxy resins produced with BPA are used as lacquers to coat metal can surfaces in contact with foods and beverages, while polycarbonate plastics are used in food and drink containers. Many studies have reported BPA migration from can surface coatings or plastics into foods and food-simulating liquids, especially at high temperatures and with repeated use of plastic products [9, 10], and from wine vats [11], canned drinks [12] and baby bottles [13]. In 2002, the European Food Safety Agency (EFSA) set a Specific Migration Limit (SML) of 3 mg bisphenol-A per kg food (3 ppm) for the protection of EU consumers. A recent re-evaluation of BPA [14] confirmed a non-observed-adverse-effect level (NOAEL) of 5 mg/kg body weight/day and established a maximum total daily intake (TDI) of 0.05 mg/kg body weight. Unfortunately, this report did not take into consideration newly discovered sources of food exposure to BPA, e.g. from recycled paper and cardboard containers [15].

Alkylphenols are also high-production volume manmade chemicals, used primarily to manufacture alkylphenol ethoxylates. Alkyphenols and their ethoxylated derivatives have been in use for over 50 years in a large number of industrial processes, including pulp and paper, textiles, coatings, agricultural pesticides, lube oils and fuels, metals and plastics [16]. The most commercially important alkylphenols are nonylphenol (NP) and octylphenol (OP), which exist in different isomers and are used to make nonylphenol and octylphenol ethoxylates. Restrictions are now in place in the EU [17] to govern the marketing and use of NP and ethoxylates. Before this measure was taken, they were used in industrial detergents, industrial processes, leather processing, paints, spermicidal lubricants, pesticide formulations, hair dyes, cosmetics and personal care products [16]. OP is mainly used to make phenolic resins (98%), with the remainder converted into ethoxylates to produce surfactants [18]. It can also be present as an impurity in NP

along with other OP isomers. Phenolic resins are used in rubber processing to make tyres (82%) and in printing inks, electrical insulation varnishes and in the production of ethoxylated resin for offshore oil recovery [18]. OP ethoxylates are mainly used in emulsion polymerisation, textile processing, water-based paints, pesticide and veterinary medicine formulations [18]. Exposure to alkylphenols can occur at the workplace or through the use of consumer products containing alkylphenols. Contamination of the environment and food chain means that the general public can also be indirectly exposed to NP *via* food and food packaging materials and, to a lesser extent, *via* contaminated drinking water [19].Thus, NPs used as additives (stabilisers, antioxidants) in plastic packaging can migrate from materials in contact with food [20–22]. In fact, a comprehensive study of German supermarket foods showed that NPs were ubiquitous in food and were not dependent on its fat content [21].

Despite the potential for human exposure created by their widespread use, only very limited data are available on the presence of BPA, NP and OP in humans [19]. NP and OP can be detected in human blood plasma and serum [22] and have been reported in sera from maternal and umbilical cord blood [23, 24]. NP has also been detected in urine [25, 26]. There are very few reports on BPA, including a recent publication by our group [27], describing its presence in 55% of adipose tissue samples from adult women. This study formed part of a wider investigation into the exposure of women in Southern Spain to organochlorine pesticides, polybrominated diphenyl ethers (PBDE), biphenyls (PBB), and polychlorinated biphenyls (PCB). The frequency and extent of exposure observed in the women of reproductive age indicated an urgent need for research into the exposure of children to these chemicals.

2 Materials

2.1 Subjects

Eighty-six adipose tissue samples were collected from children (21 girls and 65 boys) in the course of surgical treatment of benign diseases at the Virgen de las Nieves University Hospital of Granada (Spain). Parents signed informed consent to participation before undertaking a face-to-face interview with a trained researcher on their sociodemographic characteristics and life-style factors, using a structured questionnaire. The study was approved by the Ethics Committee of our institution.

2.2 Sample collection and storage

Adipose tissues were placed into a glass vial on ice, coded and frozen to -70º C, always within 30 min of being excised, and samples were stored at the same temperature at the Laboratory of Medical Investigations until their dispatch to laboratory for analysis.

2.3 Reagents and standards

All reagents were of analytical grade unless otherwise specified. Water was purified with a Milli-Q plus system (Millipore, Bedford, USA). Methanol,

hexane, ethanol, ethyl acetate, diethyl ether, dichloromethane, anhydrous sodium sulfate, o-phosphoric acid and sodium hydroxide were supplied by Panreac (Barcelona, Spain). All solvents and reagents were tested to ensure they were free of contamination from compounds. Octylphenol (OP), nonylphenol (NP), bisphenol F (BPF), BPA and tetrachlorobisphenol A (Cl_4BPA) were supplied by Sigma-Aldrich (Madrid, Spain). Monochloro-, dichloro- and trichloro-bisphenol A (ClBPA, Cl_2BPA, Cl_3BPA) were synthesized at the Department of Analytical Chemistry of our university. Stock standard solutions (100 mg/l) of each chemical compound were prepared in n-hexane and stored in dark glass bottles at 4 ºC until use, remaining stable for at least three months. These solutions were used to spike the adipose tissue samples.

2.4 Sample preparation, extraction and derivatization

Two hundred mg of adipose tissue were homogenized with 6 ml n-hexane. Then, 2 ml of acetonitrile were added to the n-hexane solution. After shaking for 3 min, the aqueous phase was separated and dried with a gentle stream of nitrogen. Prior to the extraction, adipose tissue samples were spiked with BPF as internal standard. SPE cartridges (silica-based bonded C_{18} cartridges AccuBONDII ODS-C_{18}, Agilent Technologies, Waldbron, Germany) were conditioned with 3 ml of diethylether, 3 ml of methanol, and 3 ml of deionized water on an SPE manifold at a rate of 1-2 ml/min.

Sample extracts were resuspended using 15 ml of deionized water and passed through SPE cartridges at a flow rate of 1-2 ml/min. Then, cartridges were dried under vacuum for 20 min. BPA and its chlorinated metabolites were eluted from sorbents with 3mL of a mixture of diethyl ether/methanol (9:1 v/v) at a flow rate of 1 ml/min. Finally, eluents were evaporated to dryness under a stream of nitrogen; 120 μL of ethyl acetate and 30 μL of BSTFA/TMCS (1:1, v/v) were added to the reaction vial in order to resuspend the residue and carry out the derivatization. Next, vials were closed and heated at 60 ºC for 30 min. Once the derivatization process was completed, 2 μL of the reaction mixture was injected into the gas chromatography–mass spectrometry (GC-MS) system.

2.5 Apparatus: gas chromatography–mass spectrometry analysis

GC-MS analysis was performed using a 6890 Agilent (Agilent Technologies, Wilmington, USA) gas chromatograph with a 7683 series injector and a quadruple mass filter 5976 network mass selective detector (MSD) following a previously published method [33]. In brief, a ZB-5 MS Zebron capillary column (30 m×0.25 mm i.d.; 0.25μm film thickness) from Phenomenex was used. The MSD was operated in full-scan mode from 50–550 m/z for qualitative determinations and in selected ion-monitoring (SIM) mode for quantitative determinations. The mass spectrometer was calibrated every day, using perfluorotributylamine (PFTBA) as calibration standard. HPCHEM chromatography software was used for data acquisition and integration. The injector port of the GC was set at 250ºC. The silicated samples were automatically injected using the splitless-injection mode. The transfer line of the

GC to the MS was set at 270ºC, and the electron impact (EI) ion source of the MS was set at 250ºC. The ionization energy was 70 eV. The GC oven temperature program was as follows: the initial oven temperature was set at 120ºC, held for 2 min, and then increased to 230 ºC via ramp of 30ºC/min and maintained at 230ºC for 2 min, and from 230ºC up to 270ºC via ramp of 40ºC/min and maintained at 270ºC for 6 min. The carrier gas was high-purity helium (99.999%) with a constant flow of 1ml/min. A solvent delay time of 4 min was used to protect the ion multiplier of the MS instrument from saturation. SIM mode was used to carry out measurements. SPE was carried out on a Supelco vacuum manifold for 12 columns connected to a Supelco vacuum tank and a vacuum pump.

2.6 Analytical performance

Calibration graphs for samples treated according to the above procedure were constructed using SIM mode. Linearity of the calibration graphs was tested according to the Analytical Methods Committee; the *lack-of-fit* test was applied to two replicates and two injections of each standard. The results for the intercept (a), slope (b), correlation coefficient (R^2) and probability level of the lack-of-fit test and for the linearity, precision, accuracy, sensitivity and selectivity of the overall assay have been presented elsewhere. Recoveries of tested compounds were 95-105% in all cases. The limit of detection (LOD) ranged from 0.5 ng/ml for BPA to 3 ng/ml for Cl_4BPA.

2.7 Quality control

The methodology was validated using recovery studies with spiked samples. Recoveries of OP, NP, BPA and its chlorinated derivatives ranged from 91.3 to 100.4%. BPF was selected as internal standard because this compound was efficiently extracted from adipose tissue (mean recovery: 98.4%) and did not elute with any of the evaluated chemicals.

3 Results

Mean age of the children was 4.26 yrs (Range, 1 month to 11 yrs); 24% were girls and 76% were boys. Most children (78%) were the first or second pregnancy of their mothers. Table 1 shows means, medians, and SD of NP, OP, BPA and chlorinated derivatives in samples with presence of these chemicals above the LOD. NP and OP were above LOD in 31 (36%) and 21 (24%) out of 86 samples, respectively. BPA was above LOD in 53 (62%) out of 86 samples. Among Cl(x)BPA, Cl_2BPA was the most frequent (99%) and abundant, followed by Cl-BPA (64%) and Cl4-BPA (12%).

Mean ± SD values per g of adipose tissue were 38.27 ± 56.91 for NP, 6.26 ± 6.63 ng for OP, and 17.47 ± 14.82 ng for BPA. Mean concentrations of mono-, di-, tri and tetrachloro-BPA derivatives were 7.32 ± 4.25, 14.45 ± 9.79, 4.13 ± 1.35 and 3.54 ± 1.94 ng/g of adipose tissue, respectively. A correlation was found between BPA and some chlorinated derivatives, with a significant

Spearman correlation coefficient (r) between BPA and chloro BPA (BPA/ClBPA, $r = 0.69$, $p < 0.001$). Most of the residues were higher in girls than in boys, but the difference only reached significance in the case of NP (Girls, GM = 25.04, boys 19.86, $p = 0.053$). Age emerged as a determinant of exposure (Cl2BPA/age, $r = 0.22$, $p = 0.057$), as found for other persistent and fat soluble compounds. Moreover, body fat content had an effect on the presence or concentration of these chemicals. Thus, Spearman correlation coefficients were significant between BMI and concentrations of NO, OP and tetra-chlorinated BPA (NP/BMI, $r = 0.49$, $p = 0.024$;OP(BMI, $r = -0.510$, $p = 0.054$; Cl4BPA/BMI, $r = 0.89$, $p = 0.019$).

Table 1.

	n	>LOD n(%)	Mean	Median	SD
NP	86	31 (36.05)	38.2703	21.85	56.91858
OP	86	21 (24.42)	6.2695	3.56	6.63816
BPA	86	53 (61.63)	17.4772	12.78	14.8285
Cl BPA	86	55 (63.95)	7.3262	6.39	4.25623
Cl2 BPA	86	85 (98.84)	14.4573	11.41	9.7946
Cl3 BPA	86	2 (2.33)	4.135	4.135	1.35057
Cl4 BPA	86	10 (11.63)	3.543	3.445	1.93883

4 Discussion

The absence of previous reports on BPA and alkylphenols in children's adipose tissue precludes comparisons with levels in other populations. Nevertheless, data have been published on BPA content in adipose tissue from women living in the same geographical area [27] and in breast milk and placentas from women of other regions [28, 29]. Thus, adipose tissue levels of BPA were much higher in the children (17.47 ± 14.82 ng/g) than in the women (3.16 ± 4.11 ng/g). BPA was found in the breast milk of all 23 healthy women investigated by Sun et al. [28] with a range of 0.28-0.97 ng/ml and a mean concentration of 0.61 ng/ml. Ye et al. reported a mean concentration of 1.9 ng/ml in breast milk, with free BPA in 60% and total BPA (including free BPA and BPA conjugates) in 90% of study samples [29]. BPA has also been detected in colostrum (3.41 ± 0.01 ng/ml, range 1-7 ng/ml) [30], which is less fatty than mature milk. The above data suggest that breast feeding is a source of exposure in younger children. BPA was found in placentas and maternal blood, with a mean value of 11.2 ng/g of tissue and a maximum of 104.9 ng/g in placenta compared with a mean of 4.4 ± 0.64 ng/ml in serum of these mothers [31]. Serum BPA levels in this sample of pregnant women appeared high, contrasting with findings of 1.49 ± 0.11 and 0.64 ± 0.10 ng/ml, respectively, in a population of Japanese men and women [32]. Studies have reported blood BPA concentrations ranging from below 1 ng/ml to 19 ng/ml in individuals with no intentional exposure [33–35], although this wide variation may be related to the different BPA quantification method used. These

data suggest that children may also be exposed to BPA during pregnancy. Accumulation of Cl_2BPA in the adipose tissue of children is easier to interpret than that of BPA, since there is much more information on organohalogenated compounds in adipose tissue [36-38]. In fact, results obtained in these children confirm previous data on chlorinated bisphenols in women. The main concern about the accumulation of chlorinated BPAs in fatty tissue derives from their demonstrated estrogenicity in various *in vitro* and *in vivo* models [39, 40]. All of the chlorinated bisphenols investigated here, with the exception of Cl_4BPA, induce proliferation of MCF-7 breast cancer cells in culture at concentrations of ≥ 1 mM [39]. In ovariectomized rats, Cl_2BPA mimicked BPA by increasing uterine wet weight and showed a stronger effect than BPA on the number of BrdU-positive cells in the uterine endometrium [40]. Another organohalogenated BPA derivative, fluorine-containing bisphenol-A (bisphenol-AF), is also estrogenic for MCF-7 breast cancer cells, promoting cell proliferation and increasing the synthesis and secretion of cell type-specific proteins [41, 42].

There have been few studies on human contamination by alkylphenols, but available data suggest that, as in the case of bisphenols, children are exposed before birth (detection in umbilical cord) and during breastfeeding (detection in breast milk). Thus, NP was found in human umbilical cords at a concentration of 2 ng/kg [23], demonstrating its passage *via* the placenta from the contaminated mother to the growing foetus. This was more recently confirmed in a Dutch study on chemical contaminants in human umbilical cord Netherlands [24], which detected NP in 12 out of 17 cord blood samples. A mean NP concentration of 0.3 mg/kg was reported by Guenther *et al.* [21] in breast milk of German mothers. Therefore, pregnancy and breastfeeding are emerging as putative sources for the inadvertent exposure of children to phenolic compounds with endocrine disrupting activities.

Despite the importance of the mother-child transfer of compounds, the widespread contamination of the environment and food chain makes it likely that the exposure of children to BPA and alkylphenols is mainly *via* food and food packaging materials and, to a lesser extent, *via* contaminated drinking water. BPA and alkylphenols are known to be present in products in everyday use but there is inadequate information on their levels in food and water. The finding of bisphenols and alkyphenols in the adipose tissue of children is of major concern and clearly indicates deficiencies in protection systems against exposure to endocrine disrupting chemicals.

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