Biomonitoring of bisphenol A in hair of Greek population

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Article info

Article history:
Received 25 August 2014
Received in revised form 13 October 2014
Accepted 14 October 2014
Available online 11 November 2014
Handling Editor: J. de Boer

Keywords:
Hair
Bisphenol A
LC–APCI–MS
Biomonitoring

Abstract

Objective: Bisphenol A (BPA) is considered as an endocrine-disruptor in which humans are exposed daily mainly by food-contact products, toys, recycled paper and drinking containers. In this study, we validated a method for the isolation and the detection of BPA in human head hair samples and estimated the burden of BPA in hair of Greek population. Methods: Hair samples were collected from 69 volunteers. The isolation of the BPA was performed by solid–liquid extraction with methanol and its determination by a liquid chromatography–mass spectrometry technique. Results: The limits of quantification (LOQ = 9.7 pg mg⁻¹), the accuracy (92.6%), the precision (inter 15.3%, intra 13.0%), the ion suppression (<8.1%) and the recovery (88.3%) of the method were found satisfactory. Differences in the detection rates of the positive samples as well in detected levels of BPA between rural and urban population were observed. The 41.2% of the samples collected from urban population were positive whereas the positive samples from rural population were 14.8% (p = 0.025). The mean concentration of the positive samples for the urban population was 64.1 pg mg⁻¹ (17.7–192.8 pg mg⁻¹), for the rural population 40.3 pg mg⁻¹ (13.1–72.8 pg mg⁻¹) and for the children 37.9 pg mg⁻¹ (13.1–72.8 pg mg⁻¹). Significant statistical differences (p = 0.021) were observed though between urban and rural population only when negative samples were replaced with LOD/2 values. Conclusion: The proposed method was successfully applied for the determination of BPA in hair for the estimation of the population burden to BPA.

1. Introduction

Bisphenol A (BPA), an endocrine-disruptor, has chemical structural features similar to 17b-estradiol and other natural estrogenic compounds found in food, such as daidzein – one of the major soy isoflavones (Peyre et al., 2014). Nowadays, BPA is used as a monomer in the production of polycarbonate plastics and epoxyresins and many consumer products including toys, recycled paper, drinking containers, eyeglass lenses, dental composites, medical equipments and tubing, consumer electronics and in the production of thermal paper. Its release in the food chain is related mainly by sterilization process (temperature and time) and the amount of coating. As it is mentioned, BPA migrates from the coating of the can into the content mostly during the sterilization process (Howdeshell et al., 2003; Takao et al., 2002; Geens et al., 2010; Mungua-Lopez et al., 2005). Duration of can storage, elevated temperatures during storage or damage of the can do not affect the levels of BPA in the food (Goodson et al., 2004). Moreover,
due to the low water solubility of BPA, canned food with high fat content has higher detected levels of BPA in the solid portion compared to the liquid portion (Geens et al., 2010).

Exposure to BPA has resulted to extensive human health effects, since it exhibits estrogenic activity. It seems to interfere with the production, release, transport, metabolism, binding, action or clearance of endogenous hormones involved in human conception and development, often at extremely low doses (Lathi et al., 2014).

The effects of BPA in human health have been extensively studied. Various studies indicate an association of actual BPA levels even at very low doses with cardiometabolic, disorders reproductive disorders such as endometriosis, infertility, polycystic ovary syndrome (PCOS) and thyroid disease (Melzer et al., 2010; Caserta et al., 2013; Fenichel et al., 2013; Rutkowska and Rachon, 2014).

So far, according to literature BPA, can be found in human cord blood, placenta, amniotic fluid and breast milk, resulting in the exposure of fetuses and newborns through maternal exposure, where it seems to be associated with low birth weight and reduced head circumference (Lathi et al., 2014).

European Food Safety Authority (EFSA) has permitted the use of BPA in food contact materials in the European Union (EU) under Regulation 10/2011/EU, relating to plastic materials and articles intending to come into contact with food products. In January 2011, the European Commission adopted Directive 2011/8/EU, prohibited the use of BPA for the construction of infant feeding bottles [http://www.efsa.europa.eu/en/topics/topic/bisphenol.htm, 2014]. It therefore on January 2014, recommended that the current tolerable daily intake (TDI) of BPA is 5 \( \mu g \) kg bw d\(^{-1}\).

The aim of this study was to develop and validate a liquid chromatography–mass spectrometry (LC–APCI–MS) based method for the determination of the BPA in hair matrix and its application in hair samples collected from Greek population for the estimation of the burden to BPA.

2. Materials and methods

2.1. Materials

BPA (\( >99\% \)) and methanol (LC–MS grade) were purchased by Sigma–Aldrich (St Louis, MO, USA). Phenobarbital-d\(^5\) was obtained from Isotec Inc. (Miamisburg OH, USA). Ultrapure water was produced by a Direct-Q 3UV water purification system (Merck, Germany).

2.2. Hair sampling

Head hair samples (total length, from 2 to 45 cm) were collected from 69 volunteers during 2011–2014. The examined population was divided in two groups; the urban population (\( n = 34 \)) and rural population (\( n = 35 \)). Eight (8) samples were from children (below 18 years old) and the rest (61) from adults. The hair samples were placed in an envelope and stored in a dark and dry place at room temperature until further analysis. A written informed consent was obtained from all participants.

2.3. BPA extraction from hair

Hair samples (usually 120–150 mg) were washed twice with 5 ml water (for 5 min) in an ultrasonic bath and with 2 ml of methanol for the removal of external contaminants and dried at room temperature. Then, hair was cut in smaller pieces, weighed (100 mg) and transferred to a screw-top glass tube. Fifty (50) ng of phenobarbital-d\(^5\) (IS) were added and BPA was extracted with 2 \( \times \) 2 ml of methanol by incubating hair samples at 50 ± 5 °C in an ultrasonic bath for 2 \( \times \) 2 h. The organic phases were isolated, filtered through a 0.2 \( \mu m \) membrane filter and evaporated to dryness under nitrogen stream at 35 °C. The residue was dissolved in 100 \( \mu l \) of methanol.

2.4. Chromatographic analysis

A liquid chromatography–mass spectrometry system was used for the analysis of BPA. The system is comprised of a binary LC pump (Shimadzu Prominence LC), a vacuum degasser, an autosampler and a column oven. A gradient of water (solvent A) and methanol (solvent B) were selected as the mobile phase with a flow rate of 0.6 ml min\(^{-1}\): starting at 40% solvent B (1 min), 98% solvent B (8 min linear ramp), 98% solvent B (13 min) and finally 40% solvent B (13.01 min). Separation of analytes was achieved on a Discovery C18 HPLC column (250 × 4.6 mm, 5 \( \mu m \)) thermostated at 30 °C. BPA’s retention time was 11.43 min and for IS was 9.76 min (Table 1).

A mass spectrometer (LCMS-2010 EV Shimadzu), coupled with an atmospheric pressure chemical ionization (APCI) interface and a single quadrupole mass filter was used in a selected ion monitoring (SIM) negative mode, with ions \( m/z \) 271.15, 133.00, 259.15 and 273.20 for the BPA, and 236.15 for the IS (Fig. 1). The ion with \( m/z \) 271.15 was that with the highest intensity compared to other monitoring ions (as it is depicted in Fig. 1-E). Nonetheless, the ion with \( m/z \) 133.00 provided clearer chromatograms compared to other ions and it was finally selected for the quantitation of BPA in head hair samples. The interface, CDL and heat block temperatures were 400 °C, 200 °C and 200 °C, respectively. The detector voltage was 1.5 kV, the nebulizing gas flow was 2.5 l min\(^{-1}\) and the drying gas was set at 0.02 MPa.

2.5. Working solutions, quality control and spiked hair samples

Stock solutions of BPA and IS in a concentration of 100 \( \mu g \) ml\(^{-1}\) were prepared in methanol. The working solutions of BPA (0, 25, 50, 100, 250 and 500 ng ml\(^{-1}\)) were prepared by further dilutions of stock solution. All solutions were stored at −20 °C.

Hair samples were collected from the staff of the Laboratory of Toxicology, University of Crete and analyzed for the detection of BPA. The samples that provided negative results (<LOD) were pooled, washed, dried and used as blank for the preparation of spiked samples at concentration range from 0 to 250 pg mg\(^{-1}\) (0, 10, 25, 50, 100 and 250 pg mg\(^{-1}\)) (Tsatsakis et al., 2010). The spiked samples were used for the preparation of spiked curves and furthermore for the determination of BPA levels in the collected head hair samples.

Quality control samples were prepared by immersion of blank hair in 100 ng ml\(^{-1}\) of 30 ml aquatic solution of BPA for 16 h. After that, hair was washed 10 times with water and 5 times with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Analytical parameters of the developed LC–APCI(−)–MS base method for the detection of BPA in hair samples.</th>
</tr>
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<tbody>
<tr>
<td>Target</td>
<td>Phenobarbital-d(^5) (IS)</td>
</tr>
<tr>
<td>Rt (min)</td>
<td>11.43</td>
</tr>
<tr>
<td>Target m/z</td>
<td>133.00</td>
</tr>
<tr>
<td>Q1 m/z</td>
<td>271.15</td>
</tr>
<tr>
<td>Q2 m/z</td>
<td>259.15</td>
</tr>
<tr>
<td>Q3 m/z</td>
<td>273.20</td>
</tr>
<tr>
<td>( r^2 ) (spiked)</td>
<td>0.9932</td>
</tr>
<tr>
<td>( r^2 ) (standards)</td>
<td>0.9991</td>
</tr>
<tr>
<td>LOD (pg mg(^{-1}))</td>
<td>2.9</td>
</tr>
<tr>
<td>LOQ (pg mg(^{-1}))</td>
<td>9.7</td>
</tr>
</tbody>
</table>

LOQ: limit of quantification, LOD: limit of determination.

Q1, Q2, Q3: qualification ions.
methanol for the removing of any external contamination. Totally 3 quality control samples were prepared.

3. Statistical analysis

The expression of BPA levels was in the form of mean, standard deviation and median, while discrete variables were in the form of counts and % percentages. Associations between discrete variables were tested by Pearson’s chi-square test. Differences of BPA levels were examined for positive samples and the inputted with LOD/2 values. Graphical representation was given in the form of box plots (continuous data). A level of significance was set at 0.05 and analysis of data was made using IBM SPSS Statistics 20.0.

4. Results

4.1. Optimization of BPA extraction from hair

Two approaches for the isolation of BPA from hair matrix were tested; (a) alkaline digestion and (b) methanolic extraction in ultrasonic bath. The alkaline digestion was performed by adding 1 ml of NaOH, 10 N and 1 ml of water in 100 mg hair following incubation of hair at room temperature in an ultrasonic bath for 2 h. Then, the isolation of BPA was executed by liquid–liquid extraction with $\frac{2}{3}$ ml ethyl acetate, separation of organic phase, evaporation to dryness and reconstitution in 100 μl of methanol. No results were obtained (0% recovery) at tested spiked levels 50 and 100 pg mg$^{-1}$ of BPA.

For the optimization of the extraction time and the determination of the efficiency of the methanol on the recovery of BPA, quality control samples were analyzed. Although, the control samples, which have been prepared by immersion of blank hair in 100 ng ml$^{-1}$ of 30 ml aquatic solution of BPA for 16 h, did not correspond to real contamination scenario, they can be considered as quality control samples representing authentically exposed hair samples. In 100 mg of the prepared quality control sample, 2 ml of methanol were added and sonication for 2 h followed. Methanol was collected (fragment 1), filtered, concentrated and analyzed, while another aliquot of 2 ml of methanol were added in the same samples and the above procedure was followed out twice. Totally,
6 h of extraction was performed (methanolic fragments 1, 2 and 3). In the first methanolic fragment (2 h of extraction) the mean extraction rate was 82.4 ± 14.1, in the second methanolic fragment the mean extraction rate was 14.2 ± 13.2% and in the third methanolic fragment the mean extraction rate was 3.4 ± 14.1%. Our results indicated that in a 4 h extraction procedure the achieved recovery of BPA from hair matrix was 96.6%.

4.2. Method validation

The analytical parameters of recovery, linearity, ion suppression, accuracy and precision were checked and determined.

4.3. Linearity, limits of detection and quantification

The instrument response was checked by the ratio of BPA area to IS area in standards solution and found linear from 0 to 500 ng ml\(^{-1}\) (\(r^2 = 0.9991\)). The spiked curve was estimated also by using the ratio of BPA response to IS response and was linear (\(r^2 = 0.99932\)) in the concentration range 0–250 pg mg\(^{-1}\) (Table 1). The limit of quantification (LOQ) (signal to noise ratio > 10) and the limit of detection (signal to noise ratio > 3) were determined from the spiked hair sample with the lowest concentration (10 pg mg\(^{-1}\)) and were found to be LOQ = 9.7 pg mg\(^{-1}\) and LOD = 2.9 pg mg\(^{-1}\), respectively (Table 1).

4.4. Recovery and ion suppression

The mean recovery ± SD% as well as the % recovery per tested concentration level are presented in Table 2. Recovery was calculated by spiked samples at concentration levels 25, 50, 100 and 250 pg mg\(^{-1}\). The spiked hair samples (\(n = 7\)) were extracted as previously described and analyzed. The recovery was determined as the ratio of the analyte peak area to the analyte peak area in the corresponding methanolic standard. At level of 25 pg mg\(^{-1}\), the % recovery was 94.3%, at 50 pg mg\(^{-1}\) it was 90.6%, at 100 pg mg\(^{-1}\) it was 79.05 and at 250 pg mg\(^{-1}\) it was 89.2% with a mean recovery value for all tested levels 88.3 ± 6.5%.

For the evaluation of the ion suppression, a comparison of the detected area of BPA at spiked samples (25 and 100 pg mg\(^{-1}\), \(n = 3\)) to extracted blank hair samples spiked with BPA at the same levels just before the injection into the LC-MS instrument was performed. The ion suppression was evaluated and expressed as % decreased peak response of the system. The mean % ion suppression was 8.1% at 25 pg mg\(^{-1}\) and 6.4% at 100 pg mg\(^{-1}\) (Table 2).

4.5. Accuracy and precision

The accuracy of the method was calculated from five concentration levels (10, 25, 50, 100 and 250 pg mg\(^{-1}\), \(n = 6\)) and the % accuracy values per concentration level are presented in Table 2. The mean accuracy of the method was 92.6 ± 17.5%. Both inter-days and intra-day precision were estimated at concentrations of 10, 50, 100 and 250 pg mg\(^{-1}\) (\(n = 3\)) and expressed as % relative standard deviation (% RSD). The intra-day precision was ranged from 5.3% to 18.6% for the tested spiked levels with mean value 13.0 ± 5.7% and the inter-days precision was found to range from 5.3% to 22.2% with a mean value 15.3 ± 7.1% (Table 2).

4.6. Biomonitoring of BPA in head hair samples

In total, 69 head hair samples (total length) were collected from Greek population and analyzed. The length of the samples varied from 2 to 45 cm providing information up to 45 months of exposure to BPA. Hair samples were divided in two main groups, rural and urban population as aforementioned. The 81.1% (\(n = 56\)) of the samples were collected from females and the 18.8% (\(n = 13\)) from males. The 11.6% (\(n = 8\)) of the population was children.

The 41.2% of the samples collected from the urban population were positive, whereas the positive samples from the rural population were 14.8%. The mean concentration of the positive samples for the urban population was calculated to be 64.1 ± 52.0 pg mg\(^{-1}\) (17.7–192.8 pg mg\(^{-1}\)), while for the rural population it was 40.3 ± 18.6 pg mg\(^{-1}\) (13.1–72.8 pg mg\(^{-1}\)). No statistical significant differences were observed between rural and urban population (\(p = 0.050\)). When the LOD/2 values were taken into account for the negative samples (<LOD), statistical differences (\(p = 0.021\)) were depicted between urban (mean value 27.3 ± 45.2 pg mg\(^{-1}\)) and rural population (mean value 7.2 ± 15.4 pg mg\(^{-1}\)) (Table 3, Fig. 2).

The 37.5% of samples collected from children were positive compared to 14.8% of the positive samples collected from adults of the same region (rural) or 29.5% of the adults samples collected from both examined regions (urban and rural). The mean detected concentration of BPA in children head hair was 37.9 ± 31.1 pg mg\(^{-1}\) (range 13.1–72.8 pg mg\(^{-1}\)), while the corresponding values were 40.3 ± 18.6 pg mg\(^{-1}\) for the adults of the same region and 58.5 ± 47.3 pg mg\(^{-1}\) for the adults of both examined regions. No statistical differences (\(p > 0.05\)) in the detected levels of BPA were observed in hair samples collected from children and adults, either with or without input LOD/2 values (Table 3).

5. Discussion and conclusion

BPA has been measured in different biological matrices (urine, amniotic fluid, breast milk) (Edlow et al., 2012; Mendonça et al., 2014; Zhou et al., 2014). In recent years, Bisphenol S (BPS) and Bisphenol F (BPF) are replacing BPA in the production of some products. Zhou et al. studied the concentrations of the bisphenol analogs in urine samples and found that BPA was the most frequent analog with median concentration higher (95%, 0.72 ng ml\(^{-1}\)) than BPS (78%, 0.13 ng ml\(^{-1}\)) and BPF (55%, 0.08 ng ml\(^{-1}\)) (Zhou et al., 2014). Kubwabo et al. measured the levels of total maternal BPA and free BPA in urine samples of pregnant women in the second and third trimester of gestation with detection rates 86% for conjugated BPA and 22% for free BPA (Kubwabo et al., 2014). In a similar study,
by Edlow and co-authors, total BPA was detected in 16/20 third trimester amniotic samples, instead of free BPA that was detected in 9/20 of them. In contrast, total BPA was present only in 2/20 samples collected in the third trimester (Edlow et al., 2012). An in vitro study showed that human endometrium accumulates BPA and maybe releases BPA to the fetal compartment during pregnancy through the placenta (Mannelli et al., 2014). These findings are in agreement with an ex vivo study that indicated a rapid materno-fetal transfer of BPA across the placenta when the concentration of BPA are low (Balakrishnan et al., 2010).

In another study concerning the levels of BPA in urine samples from 105 children, the given result was that 95% were positive for BPA providing higher concentration of BPA between obese children compared to normal weight children. Moreover the concentration of BPA in the obese boys exceeded more than 20% of the detected levels compared to obese girls. In the latter, no differences were found between the two sexes for normal weight children (Nicolucci et al., 2013).

High frequencies of positive urine samples were also reported (99.8%) for Korean adult population (Kim et al., 2011). No significant differences were reported between sex and other sociodemographic factors (income, BMI, smoking status) except the place of residence that a statistical significant association (p < 0.001) was found between rural and urban population (higher levels of BPA for rural population). In contrast, in the study of Calafat et al. higher levels of BPA were reported in females than to males (p = 0.04), as well as in children to adolescents (p < 0.001) and in children/adolescents to adults (p = 0.003) (Calafat et al., 2008). Aforementioned study was in agreement with a subsequent study that reported measured levels of BPA in premature infants higher than the general population (Calafat et al., 2009). On the other hand, as it is mentioned by Wang (Wang et al., 2014) the highest levels of BPA in urine were found in children at the age of 12 years with geometric mean concentration of 1.55 ng ml\(^{-1}\), followed by the age of 11 years with 1.18 ng ml\(^{-1}\), 10 years with 1.05 ng ml\(^{-1}\) and 9 years with 0.99 ng ml\(^{-1}\) and no significant differences in urinary BPA levels were found between obese and normal weight children.

To the best of our knowledge, this is the first reported methodology concerning detection of BPA in hair. This method was successfully applied for the determination of the BPA in hair in order to estimate the total burden of Greek populations (rural and urban) to BPA. Differences in detection rates and in detected levels of BPA between the examined populations were observed. In contrast to a previous published study (Kim et al., 2011) the examined urban population of this current study seems to be more burdened compared to rural population (higher detection rates and higher detection levels). No significant differences were observed between children and adults and this could be possibly explained due to the small number of samples of the children’s group in comparison to the adult groups. Besides that, the main purpose of this study was to develop, validate and apply of a new methodology for the detection of BPA in human hair for the assessment of the chronic and long-term exposure to it. This ability can be used in future biomonitoring studies concerning this compound of great toxicological concern.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.
Acknowledgment

The authors wish to thank Alexander I. Vardavas, for his assistance in the manuscript preparation.

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Addit. Contam. 21, 1015–1026.


