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Short Communication

Background bisphenol A in experimental materials and its implication to low-dose *in vitro* study

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ABSTRACT

In vitro low-dose studies are important to understand the mechanisms of bisphenol A (BPA) action. BPA doses used in current *in vitro* studies varied considerably, and doses as low as 10^{-15} M have been reported. The actual doses of BPA used in the *in vitro* low-dose studies were rarely checked analytically, and the background BPA levels in experimental materials, which will determine the lowest BPA dose to be used, should be investigated or considered. In this study, the background BPA levels in various materials typically used in *in vitro* low-dose studies for BPA were investigated. Background BPA levels from the use of disposable pipettes and pipette tips were low (<0.20 ng mL^{-1} or 0.88 nM). BPA was also detected in several commercial buffer solutions at levels close to the method limit of quantification (LOQ) (0.02 ng mL^{-1}; 0.088 nM). However, BPA was detected in all cell culture media obtained from various sources at levels ranging from 0.080 to 4.26 ng mL^{-1} (or 0.35 to 19 nM) with an average of 0.83 ng mL^{-1} (3.5 nM). We suggest that culture media used for low-dose BPA studies should be analysed for background BPA levels prior to use, and the medium with the lowest BPA levels should be used.

1. Introduction

In vitro low-dose studies are important to understand the mechanisms of bisphenol A (BPA) action. For *in vivo* toxicological studies, levels of BPA below the current lowest observed adverse effect level (LOAEL) of 50 μg kg^{-1} d^{-1} established by the United States Environmental Protection Agency (1993) were considered as low-dose, while low-dose concentration for *in vitro* cell or organ culture studies on BPA is generally defined as less than the LOAEL cut-off value of 50 ng mL^{-1} or 0.219 μM (2.19×10^{-7} M) (Welshons et al., 2006; Wetherill et al., 2007). Levels of BPA doses used in the published *in vitro* studies varied considerably, and doses far below the 10^{-7} M level (even as low as 10^{-15} M) have been used in some studies (Xu et al., 2002; Watanabe et al., 2003; Satoh et al., 2004; Buterin et al., 2006; Miyatake et al., 2006; Yamaguchi et al., 2006; Hugo et al., 2008; Kochukov et al., 2009; LaPensee et al., 2009). Conclusions from the low-dose *in vitro* BPA studies, especially those in which <10^{-9} M BPA were used, are not always consistent and reproducible, and validity of results from such studies is thus unknown.

The actual doses of BPA used in the low-dose studies were rarely confirmed analytically. It is generally assumed that low BPA levels can be achieved by infinite serial dilutions of the standard solution. Although this is achievable in theory, in reality, the lowest BPA doses achievable will depend on the background BPA levels in various materials used in the experiments. BPA is used in the production of polycarbonate plastics and epoxy resins, and can migrate from these materials into any media which they contain (Le et al., 2008). Thus, information on background BPA levels in experimental materials is essential for researchers to determine the lowest BPA levels able to be used for *in vitro* low-dose studies.

During the early stages of our *in vitro* low-dose study on BPA, background BPA levels in the media and some other experimental materials were measured using the semi-quantitative enzyme-linked immunosorbent assay (ELISA) method, and BPA was detected in some of the materials. In order to confirm these results, a systematic study was conducted to investigate the background BPA levels in various media and experimental materials typically used in *in vitro* low-dose BPA studies using a much more sensitive analytical method based on gas chromatography–mass spectrometry (GC–MS). Information generated from this work will be very valuable to researchers who plan to conduct *in vitro* low-dose BPA studies in the future, as well as to regulatory scientists who may use published *in vitro* data for risk assessment purposes.

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2. Experimental procedures

2.1. Materials, chemicals, and reagents

Acetonitrile (HPLC grade), methanol (HPLC grade) were purchased from J.T. Baker (Phillipsburg, N.J.). Toluene (glass distilled), acetic anhydride (ACS grade), H_3PO_4 (85% HPLC grade), bisphenol A (99%), bisphenol A-d_{14} (98%), isooctane (pesticide-residue grade), MTBE (methyl t-butyl ether, 99.9%), Na_2SO_4 (anhydrous, ACS grade), 1-pentanol (99%), dodecane (99%) were purchased from Sigma–Aldrich (Oakville, ON). Potassium carbonate (ACS grade) and Na_2HPO_4 (ACS grade) were purchased from Fisher (Ottawa, ON).

The 50-place stirring block was obtained from Barnstead (Dubuque, Iowa). The 13 × 100 mm, 20 × 150 mm, and 16 × 100 mm disposable glass tubes were purchased from VWR (Montréal, QC). The 15-mL centrifuge tubes were purchased from Fisher (Ottawa, ON). The 22-mL vials and 6 mL glass columns were obtained from Supelco (Oakville, ON). The C18 SPE cartridges were purchased from Varian (Mississauga, ON.).

BPA and BPA-d_{14} standard solutions were prepared in acetonitrile, and stored at 4 °C. Phosphate buffer (pH 7) was prepared by dissolving 14.3 g of Na_2HPO_4 in 1 L of tap water; and the pH was adjusted to 7.0 ± 0.2 with H_3PO_4. A 1.0 M K_2CO_3 solution was prepared by dissolving 69 g of anhydrous K_2CO_3 in 500 mL H_2O. The keeper solution, used to minimize the loss of derivatized BPA during the concentration process, was a 50:50 v/v mixture of 1-pentanol and dodecane.

2.2. Sample extraction and derivatization

The sample extraction and derivatization procedures were described previously (Cao et al., 2009). Up to 10 mL of sample solution in water was transferred into a 70 mL tube. The sample was spiked with 10 μL of 5 ng μL^{-1} BPA-d_{14} internal standard solution and mixed. Fifty-five millilitres of pH 7.0 buffer solution was added to each tube, the tube was capped, and the contents were vortexed. The sample was then poured into a C18 SPE cartridge (Varian) which was previously conditioned with 13 mL of methanol and 13 mL of H_2O. Absorption was allowed to take place without vacuum. The cartridge was then rinsed with 6.5 mL of H_2O and 13 mL of 30% (v/v) MeOH in H_2O and the eluate was discarded. The C18 cartridge was eluted with 6.5 mL of 50% acetonitrile in water; the eluate was collected in a 16 × 100 mm glass tube. The eluate was vortexed, and concentrated to about 3 mL using a nitrogen evaporator.

The concentrated aqueous extract was transferred to a 22 mL amber vial, and a small stirring bar was added. Ten millilitres of 1.0 M K_2CO_3 solution and 200 μL of acetic anhydride were added to each vial. All sample vials were placed into the 50-place stirring block and stirred at low speed. Another 200 μL acetic anhydride was added after 5 min and the stirring continued for a further 10 min. Five millilitres of isooctane was added to the vial. The pH of the sample extracts was checked using a pH indicator strip, and adjusted to ensure that the pH was above 10. If pH adjustment was required, an additional 0.5 mL of 3 M K_2CO_3 solution was added. One hundred microlitres more acetic anhydride was then added and the extract stirred for another 10 min. The stirring was then stopped and the two phases allowed to separate for approximately 10 min. If there was an emulsion, the sample was split into two vials, diluted with H_2O, and re-extracted using more isooctane. The isooctane phase from the 22 mL amber vial was transferred to a glass column packed with anhydrous Na_2SO_4. The aqueous phase in the 22 mL vial was re-extracted with 5 mL of methyl t-butyl ether (MTBE) by stirring for at least 10 min at high speed. The MTBE phase was transferred to the Na_2SO_4 column. The dry organic extract was transferred to a 13 × 100 mm disposable glass tube and 30 μL of keeper solution (50:50 mixture of 1-pentanol and dodecane) was added. The sample extract was evaporated to almost dryness at 40 °C for about 45 min., using the nitrogen evaporator. The extract was then reconstituted with 220 μL of toluene, vortexed for 30 s and transferred to a GC vial containing an insert for analysis.

2.3. GC–MS analysis

An Agilent 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MSD) was used for the analysis. The flow rate of the helium carrier gas was 1.2 mL min^{-1}. The injector temperature was 280 °C. One microliter of sample extract was injected into the GC system in splitless mode. The analytes were separated on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The GC oven temperature program was set at an initial temperature of 100 °C for 1 min., raised to 225 °C for 5 min at 20 °C min^{-1}, then raised to 325 °C at 35 °C min^{-1}, and held for 1 min. The MSD was operated with electron impact ionization in selected ion monitoring (SIM) mode. The following ions were selected for BPA: m/z 213, 228, 270, 312, and for BPA-d_{14}: m/z 224. Dwell time was 35 ms for each ion. The GC–MSD interface and MSD source temperatures were 280 and 230 °C, respectively. Confirmation of BPA identity was based on the retention time and the ion ratios. The calculation of BPA concentrations in samples was based on the calibration curves of peak area ratios of the quantification ion of BPA (m/z 228) over the internal standard peak area plotted with the ratios of native BPA concentration over the internal standard concentration. Two method blanks (Milli-Q de-ionized water) were analysed in every extraction batch, and the average method blank BPA level was subtracted from the results of all samples.

3. Results and discussion

BPA is a relatively polar chemical and can be adsorbed onto the surface of undeactivated glassware by forming hydrogen bonds between BPA's hydroxyl (—OH) groups and the silanol (SiOH) groups on glass surfaces, and this effect is especially significant for BPA at low concentrations (10^{-8}–10^{-9} M and below). Thus, lab materials made of non-polycarbonate plastics, such as polystyrene and polypropylene, are preferred in low-dose BPA studies. Although BPA is not used in the production of polystyrene and polypropylene, it may be present on the surfaces of experimental materials made of polystyrene and polypropylene due to contamination during manufacture. In order to investigate the background BPA levels of various plastic items used in tissue culture studies, disposable polystyrene pipettes of various sizes, and disposable polypropylene pipette tips from different companies were soaked in 11 mL of de-ionized water contained in 15 mL polypropylene centrifuge tubes at room temperature for 24 h, and the water solutions were analysed for BPA. BPA was not detected in the de-ionized and distilled (Mili-Q) water used in testing. The method limit of quantification (LOQ) was 0.02 ng mL^{-1} (or 0.088 nM), estimated as 10 times the signal to noise ratio. The results are shown in Table 1. BPA concentrations in the water solutions from the disposable pipettes were low, close to the LOQ, ranging from 0.057 to 0.088 ng mL^{-1} or 0.25 to 0.39 nM, while BPA levels in the water solutions from the pipette tips were slightly higher (0.12–0.24 ng mL^{-1} or 0.53–1.1 nM). The background BPA observed is not expected to be due to the migration from the polystyrene and polypropylene pipettes and pipette tips, since they do not contain BPA in the first place and even BPA migration from polycarbonate is extremely

Table 1
Background BPA levels in the soaking solution of disposable pipettes and pipette tips after incubation in distilled and de-ionized water at room temperature for 24 h.

Material	Source	Volume	BPA concentration (ng mL^{-1})	BPA concentration (nM)
Polystyrene pipette	Company A	10 mL	0.088	0.39
	Company B	5 mL	0.057	0.25
	Company C	5 mL	0.059	0.26
	Company D	5 mL	0.057	0.25
Polypropylene pipette tip	Company E	1000 μL	0.15	0.66
	Company F	1000 μL	0.12	0.53
	Company E	300 μL	0.17	0.75
	Company E	200 μL	0.24	1.1
	Company E	10 μL	0.13	0.57

Table 2
BPA levels in commercial buffers.

Buffer solution	BPA concentration (ng mL^{-1})	BPA concentration (nM)
Commercial PBS (phosphate buffered saline) 10X	<0.020	<0.088
Commercial PBS 1X	0.034	0.15
Commercial lysis buffer	0.061	0.27

slow at room temperature. The most likely source for the background BPA on plastic labware is contamination during manufacture.

The background BPA levels in the buffer solutions obtained from various sources were also very low as shown in Table 2, close to the LOQ (0.02 ng mL^{-1}).

Polystyrene plates and flasks, upon which cells are cultured, were also investigated for any possible effects on background BPA levels. Media for culturing pancreatic cells was incubated in a 10 cm culture dish, a 24 well culture plate, and a T-25 culture flask, at 37 °C for 48 h and were analysed for BPA. As shown in Table 3, BPA was detected in the fresh pancreatic medium at 0.39 ng mL^{-1} (1.7 nM), and BPA levels in the media after being incubated at 37 °C for 48 h in the plates and flask were slightly lower than that in the original medium, ranging from 0.29 to 0.34 ng mL^{-1} (or 1.3 to 1.5 nM), possibly due to the adsorption onto the plates and flasks. This is contrary to the previous study by Biswanger et al. (2006) in which BPA contamination from the

Table 3
BPA levels in a commercial culture medium (with phenol red) for human pancreatic cells after being incubated in different culture containers at 37 °C for 48 h.

Sample description	BPA concentration (ng mL^{-1})	BPA concentration (nM)
No incubation	0.39	1.7
Incubation in a 10 cm polystyrene culture dish	0.29	1.3
Incubation in a 24 polystyrene well culture plate	0.33	1.4
Incubation in a T-25 polystyrene culture flask	0.34	1.5

Table 4
BPA levels in some commercial media and sera.

Samples	Sample source	BPA concentrations (ng mL^{-1})	BPA concentrations (nM)
Culture medium (with phenol red) for human whole fetal fibroblasts	Company A	0.21	0.92
Culture medium (with phenol red) for human pancreatic cells	Company B	0.39	1.7
Culture medium (with phenol red) for human coronary artery endothelial cells	Company C	4.3	19
Culture medium (with phenol red) for human fetal lung fibroblasts	Company A	0.35	1.5
Culture medium (with phenol red) for culturing human pancreatic cells (new packaging)	Company B	0.49	2.2
Culture medium (without phenol red) for human fetal lung fibroblasts	Company C	0.41	1.8
Culture medium (without phenol red and serum) for human fetal lung fibroblasts	Company C	0.10	0.44
FBS	Company C	1.9	8.2
Charcoal stripped FBS	Company D	0.11	0.48
FBS	Company D	0.080	0.35

culture dishes under typical cell-culture conditions was not observed. This is likely due to the higher detection limit (not provided) of the method they used; the lowest concentration of BPA used in their calibration curve was 20 nM (4.5 ng mL^{-1}). Even if the detection limit is ten times lower than 20 nM, it is still higher than the BPA levels found in this part of our study (<2 nM).

Since BPA was detected in the pancreatic medium, a few other commercial media and fetal bovine sera (FBS) from different sources were also analysed for BPA. BPA was detected in all media tested as shown in Table 4. Although BPA levels in most of the media are below 1 ng mL^{-1} (0.080–0.49 ng mL^{-1}, or 0.35–2.2 nM), a medium used for culturing human coronary artery endothelial cells (HCAEC) had the highest BPA level of 4.3 ng mL^{-1} or 19 nM, followed by a commercial FBS at 1.9 ng mL^{-1} or 8.2 nM. Similar BPA levels were detected in a commercial charcoal stripped serum and a non-charcoal stripped serum from the same source. The exact reasons for the relatively high BPA levels in the media or FBS are not known, possibly due to the storage of the media in the polycarbonate containers or containers with epoxy resin coatings, especially if above room temperature.

4. Conclusions

While the *in vitro* low-dose studies are important to understand the mechanisms of BPA action, background BPA levels must be considered when deciding on the lowest BPA dose levels to be used. Results from this study demonstrated that the background BPA levels in commercial culture media varied considerably, ranging from 0.08 to 4.3 ng mL^{-1} (0.35–19 nM) with an average level of 0.83 ng mL^{-1} (3.6 nM). This means that for *in vitro* low-dose studies on BPA, special measures are needed to reduce background BPA levels for dose levels of BPA less than 1 nM (10^{-9} M). Background BPA levels in culture media should be measured prior to initiation of studies to determine the *in vitro* effects of BPA at doses lower than 20 nM.

References

Biswanger, C., Davis, L., Roberts, R.A., 2006. Estrogenic impurities in tissue culture plastic ware are not bisphenol A. In Vitro Cell. Dev. Biol.-Animal 42, 294–297.

Buterin, T., Koch, C., Naegeli, H., 2006. Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis 27, 1567–1578.

Cao, X.-L., Corriveau, J., Popovic, S., 2009. Levels of bisphenol A in canned soft drink products in Canadian markets. J. Agric. Food Chem. 56, 7919–7924.

Hugo, E.R., Brandebourg, T.D., Woo, J.G., Loftus, J., Alexander, J.W., Ben-Jonathan, N., 2008. Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. Environ. Health Perspect. 116, 1642–1647.

Kochukov, M.Y., Jeng, Y.-J., Watson, C.S., 2009. Alkylphenol xenoestrogens with varying carbon chain lengths differentially and potently activate signalling and functional responses in GH3/B6/F10 somatomammotropes. Environ. Health Perspect. 117, 723–730.

LaPensee, E.W., Tuttle, T.R., Fox, S.R., Ben-Jonathan, B., 2009. Bisphenol A at low nanomolar doses confers chemoresistance in estrogen receptor-a-positive and –negative breast cancer cells. Environ. Health Perspect. 117, 175–180.

Le, H.H., Carlson, E.M., Chua, J.P., Belcher, S.M., 2008. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. Toxicol. Lett. 176, 149–156.

Miyatake, M., Miyagawa, K., Mizuo, K., Narita, M., Suzuki, T., 2006. Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol A in neurones and astrocytes. J. Neuroendocrinol. 18, 434–444.

Satoh, K., Ohyama, K., Aoki, N., Lida, M., Nagai, F., 2004. Study on anti-androgenic effects of bisphenol A diglycidyl ether (BADGE), bisphenol lA diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. Food Chem. Toxicol. 42, 983–993.

United States Environmental Protection Agency (US EPA). Bisphenol A. (CASRN 80-05-7), Integrated Risk Information System (IRIS), 1993. <http://www.epa.gov/iriswebp/iris/subst/0356.htm> (accessed 26.02.2010).

Watanabe, H., Adachi, R., Kusui, K., Hirayama, A., Kasahara, T., Suzuki, K., 2003. Bisphenol A significantly enhances the neutrophilic differentiation of promyelocytic HL-60 cells. Int. Immunopharmacol. 3, 1601–1608.

Welshons, W.V., Nagel, S.C., vom Saal, F.S., 2006. Large effects from small exposures III endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147, S56–S69.

Wetherill, Y.B., Akingbemi, B.T., Kanno, J., McLachlan, J.A., Nadal, A., Sonnenschen, C., Watson, C.S., Zoeller, R.T., Belcher, S.M., 2007. *In vitro* molecular mechanisms of bisphenol A action. Reprod. Toxicol. 24, 178–198.

Xu, J., Osuga, Y., Yano, T., Morita, Y., Tang, X., Fujiwara, T., 2002. Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells. Biochem. Biophys. Res. Commun. 292, 456–462.

Yamaguchi, H., Zhu, J., Yu, T., Sasaki, K., Umetsu, H., Kidachi, Y., 2006. Low-level bisphenol A increases production of glial fibrillary acidic protein in differentiating astrocyte progenitor cells through excessive STAT3 and Smad1 activation. Toxicology 226, 131–142.