The effect of bisphenol A and chlorinated derivatives of bisphenol A on the level of serum vitellogenin in Japanese medaka (Oryzias latipes)


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Abstract 2,2-bis (4-hydroxyphenyl) propane or Bisphenol A (BPA), has been reported to behave as an endocrine disrupter below acute toxic levels, and is widely present in the water environment. Although BPA is easily chlorinated, very little is reported on the effect of chlorinated BPA to the aquatic organisms. In this study, the estrogenic activities of BPA and its chlorinated derivatives were evaluated by the induction of vitellogenin (VTG) in the serum of mature male Japanese medaka. In addition, the effect of sodium hypochlorite on the decomposition of BPA was tested. The relative potencies of estrogenic activities of chlorinated BPA descended in the order 3,3′-diClBPA> BPA ≥ 3-ClBPA > 3,3′,5-triClBPA, and no estrogenic activity was observed in 3,3′,5,5′-tetraClBPA. Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) for both 3-ClBPA and 3,3′-diClBPA were 500 µg/L and 200 µg/L, respectively. LOEC for 3,3′,5-triClBPA was >500 µg/L. When BPA was reacted with sodium hypochlorite (24 hours; residual chlorine at 1 ppm), however, complete decomposition of BPA and its chlorinated derivatives was observed. The decrease in BPA and its chlorinated derivatives paralleled the decrease in estrogenic potency evaluated by the induction of vitellogenin (VTG) in the serum of mature male Japanese medaka.

Keywords Bisphenol A; chlorinated bisphenol A; endocrine disrupters; Japanese medaka; vitellogenin

Introduction Bisphenol A (BPA) has been reported to behave as an endocrine disrupter below acute toxic levels (Milligan et al., 1998; Schafer et al., 1999; Christiansen et al., 2000; Lindholm et al., 2000; Nishihara et al., 2000; Yokota et al., 2000; Ishibashi et al., 2001; Miller et al., 2001; Sohoni et al., 2001; Kang et al., 2002). An enormous volume of BPA has been synthesized to be used as raw material for various types of plastics such as polycarbonate resin, epoxy resin, phenol resin, plastic polyester, antioxidant, and polyvinyl chloride stabilizer. Thus, it has been detected in various types of water: in influents and effluents of sewage treatment plants, in raw and purified waters of water treatment plants, and in environmental waters.

Surveys conducted by the Ministry of Health, Labour and Welfare, Japan indicated that BPA was detected in 44% of the raw water and 8% of the purified water of 25 water treatment plants (Kunikane, 1999). Influenst and effluents of sewage treatment plants in Japan also had been surveyed regarding the concentration level of BPA during 1998–2000 by the Ministry of Land, Infrastructure and Transportation, Japan. BPA was consistently detected for influents and effluents of sewage treatment plants with the median concentration of 0.53 µg/L for influents (Ministry of Land, Infrastructure and Transportation, 2001).

The BPA present in water purification and sewage treatment plants, though in low concentration, could thus be easily chlorinated during the disinfectant processes, and the ecological effect of its chlorinated derivatives within the water environment is concerning.
Tabata et al. (2003) reported that the estrogenic activity of BPA was decreased by chlorination treatment. However, several studies have shown that estrogenic potencies of chlorinated BPA were greater than BPA in vitro (Aizawa, 2002; Fukazawa et al., 2002). Thus, it is essential to evaluate the estrogenic activity of BPA in vivo, in which toxicokinetic-based evaluation could be assessed.

Vitellogenin (VTG), the serum phospholipoglycoprotein precursor to egg yolk protein, is normally produced only in females in response to estrogens from ovaries (a female-specific protein) in the oviparous vertebrate. However, even in the case of the male when exposed to the estrogens or environmental estrogens, VTG is synthesized in the liver and secreted into the blood. Therefore, serum VTG in male fish has been used as a useful biomarker for environmental estrogens in the aquatic environment (Purdom et al., 1994; Heppell et al., 1995; Sumpter and Jobling, 1995; Palmer, 1996). Thus, the objective of this study was to evaluate the estrogenic potencies of BPA and its chlorinated derivative, using VTG induced in male Japanese medaka, Oryzias latipes, as a biomarker.

Methods

Fish

Japanese medaka, Oryzias latipes, which is recommended as estrogenic chemicals testing organisms by OECD, was used in this study. First, mature male medakas, between 3 to 5 months after hatching at our laboratory, were selected from breeding stock. Then, the selected males were reared further for at least three weeks.

Chemicals and reagents

The chlorinated derivatives of BPA were synthesized by reacting BPA with sodium hypochlorite, and the products were isolated by column chromatography (Fukazawa et al., 2001). The synthesized derivatives of BPA are 2-(3-chloro-4-hydroxyphenyl)-2-(4-hydroxyphenyl)propane (3-ClBPA), 2,2-bis(3-chloro-4-hydroxyphenyl)propane (3,3′-diClBPA), and 2-(3,5-dichloro-4-hydroxyphenyl)-2-(3-chloro-4-hydroxyphenyl)propane (3,3′,5-triClBPA). 2,2-Bis(3,5-dichloro-4-hydroxyphenyl)propane (3,3′,5,5′-tetraClBPA) was obtained from Tokyo Kasei Kogyo Co., Ltd, and BPA from Kantoh Chemicals (Figure 1). Other reagents used in this study were purchased from Wako Pure Chemicals Industries, Ltd.

Water flow-through chemical exposure system

The medaka were exposed to test chemical compounds by a water flow-through chemical exposure system (Figure 2). To avoid contamination, the room air was circulated through an activated carbon filter. Also, the exposure water tank was made entirely of glass. To avoid any effects from chemicals other than the tested compounds, the part of the set up, which comes into contact with water, was made of glass, Teflon®, silicone and stainless-steel components. The test water in each exposure chamber was renewed more than 5 times a day.

Exposure to test water and conditions

The serum VTG concentration of a portion randomly selected medaka was measured before they were exposed to test water, to ascertain the background level. Medaka were placed in the exposure tank and fed with brine shrimp (Artemia salina) 3 times daily. The water temperature was maintained at 25 ± 1°C, and light control was set at 12L: 12D. The pH and DO (dissolved oxygen) was checked once a week, and were within adequate levels for this exposure experiment.

Forty mature male medaka were subjected to one exposure experiment per test chemical compound, for five weeks, and VTG concentration was measured periodically. The
concentration of each chemical during exposure experiment was 100, 200, 500, and 1,000 µg/L for BPA, 100, 200, and 500 µg/L for 3-ClBPA, 100, 200, 500, and 1,000 µg/L for 3,3′-diClBPA, 200 and 500 µg/L for 3,3′,5-triClBPA, and 1,000 µg/L for 3,3′,5,5′-tetraClBPA. The concentration of test chemical compounds in test water was analyzed once a week. The average test chemical compounds concentrations were maintained within 77.2 to 102.6% of the preset value. The test compounds were not detected from the control test water throughout the experiment.

Collection of serum
More than five medakas were randomly selected from each test group after 3 days, 1 week, 2 weeks, 3 weeks and 5 weeks of exposure. The tail fin of the selected fish was cut, and blood was sampled with a glass capillary tube under ice-cooled anesthesia. The collected blood was centrifuged under 13,000 g, 10 min and 4°C, to separate serum and stored in a freezer (−80°C) until VTG analysis.

VTG analysis
VTG concentration in the serum was analyzed using Medaka vitellogenin ELISA (the enzyme-linked immunosorbant assay) Kit (Metocean Environment Inc.; Transgenic Inc.).
Minimum limit of determination (LOD) of VTG concentration in the serum was 200ng/mL. When VTG concentration was less than the minimum LOD, the half-numerical value of the minimum LOD was used for the calculation of mean, standard deviation.

Chlorination of BPA by sodium hypochlorite
Sodium hypochlorite, four times the BPA concentration and 1/5 the concentration of BPA, was added to determine the chlorination processes of BPA. BPA, chlorinated derivatives of BPA, and thirteen chlorophenols (ClPh) were analyzed at 5 minutes, 10 minutes, 30 minutes, 60 minutes, 120 minutes, 360 minutes, and 24 hours interval, during the experiment. The samples were first adjusted to pH 2, extracted with dichloromethane, subjected to derivatization by \( N,O \)-bis(trimethylsilyl) trifluoroacetamide (BSTFA), and then analyzed by GC/MS (gas chromatography/mass spectroscopy). The thirteen chlorophenols (ClPh) analyzed are 2-ClPh, 3-ClPh, 4-ClPh, 2,5-/3,5-diClPh, 2,6-diClPh, 2,4-diClPh, 2,3-diClPh, 3,4-diClPh, 2,4,6-triClPh, 2,3,5-triClPh, 2,3,6-triClPh, 2,3,4,5-tetraClPh, and 2,3,4,6-tetraClPh.

Statistical analysis
Data is expressed as average ± standard deviation. Since the serum VTG concentration results include LOD values, the non-parametric Mann-Whitney’s U-test was used for the statistical analysis.

Results and discussion
Serum VTG concentration
The serum VTG concentration of medaka exposed to BPA and chlorinated derivatives of BPA is shown in Figures 3 to 7. When exposed to 500 µg/L BPA and 1,000 µg/L BPA, VTG increased significantly from after 3 days and continued increasing with time. VTG increased by several thousands-fold for 500 µg/L BPA exposure (4.90 × 10^5 ng/mL) and by several ten thousands-fold for 1,000 µg/L BPA exposure (7.52 × 10^6 ng/mL) compared to control. When exposed to 200 µg/L BPA, a significant increase was observed after three weeks. However, since no significant VTG increase was observed after 3 days, 1 week, and 5 weeks, we have affirmed that the VTG induction after 3 weeks was not due to BPA exposure.

A significant VTG increase was observed after the first week of exposure to 500 µg/L 3-ClBPA, and the VTG increased by several hundred-fold than the control after the 2 weeks exposure (3.05 × 10^4 ng/mL). The VTG, compared to control, increased significantly after 3 days of 500 µg/L 3,3′-diClBPA exposure, and continued increasing with increasing exposure period and increased by several ten thousands-fold by the end of the second week of exposure (1.36 × 10^7 ng/mL).

Also, VTG increased significantly after 3 days of 1,000 µg/L 3,3′-diClBPA exposure by several hundred thousands-fold, compared to control. Mortality rate was approximately 73% after 3 days exposure in 1,000 µg/L, and approximately 17% after 2 weeks exposure in 500 µg/L. Mortality rate of the control was 0% in either case. When medakas were exposed to 500 µg/L 3,3′,5-triClBPA, VTG was detected from several fishes after 5 weeks of exposure, and no significant difference was observed, compared to control. Therefore, the estrogenic activity of 3,3′,5-triClBPA is deemed to be relatively weak. As for 3,3′,5,5′-tetraClBPA, no VTG was observed, even after 5 weeks exposure at 1,000 µg/L BPA. Thus, LOEC and NOEC were estimated to be 500 µg/L and 200 µg/L, for both 3-ClBPA and 3,3′-diClBPA. LOEC of 3,3′,5-triClBPA was >500 µg/L. The relative estrogenic potencies of these chemicals appeared in the order of 3,3’-diClBPA>BPA≥3-ClBPA>3,3′,5-triClBPA (Figure 8).
The chlorinated BPA, besides 3,3′,5,5′-tetraClBPA, were observed to have estrogenic activity. Among these derivatives, 3,3′-diClBPA resulted in stronger estrogenic activity and acute toxicity than BPA. According to some reported results on in vitro studies (Aizawa, 2002; Fukazawa et al., 2002), the estrogenic potencies of chlorinated BPA was greater than that of BPA. However, in this study, chlorinated BPA, besides 3,3′-diClBPA, resulted in weaker estrogenic activity than BPA.

Chlorination of BPA by sodium hypochlorite

Reaction of sodium hypochlorite with BPA at chlorine:BPA concentration = 4:1 or 1:5, at ambient temperature and pH 7.0 for various reaction time, produced 3-ClBPA, 3,3′-diClBPA (not detected at chlorine:BPA concentration =1:5), 3,3′,5-triClBPA and 3,3′,5,5′-tetraClBPA as indicated in Tables 1 and 2. When a solution containing 200 mg/L
BPA was chlorinated at chlorine:BPA concentration = 4:1 at ambient temperature for 10 min, formed 3-ClBPA, 3,3′,5-triClBPA, and 3,3′,5,5′-tetra ClBPA were 1.4%, 1.45%, and 55.2% of the initial BPA (weight percent: chlorinated by-product/BPA 100), respectively. After 30 and 60 minutes, 3,3′,5,5′-tetraClBPA was detected by 5.85% and 1.6% of the original BPA concentration. Original BPA was not detected after 10 minutes, and the chlorinated BPA was not detected after 120 minutes. Among chlorophenols, only 2,4,6-trichlorophenol (2,4,6-triClPh) was detected at a low concentration (0.1–0.75% of the original BPA concentration), after 10, 30, 60, and 120 minutes of reaction. Even when male Japanese medaka was exposed to 100 µg/L 2,4,6-triClPh for 5 weeks, no significant induction of VTG was observed.

When BPA was reacted with sodium hypochlorite, at 1/5 the BPA concentration, 3-ClBPA, 3,3′-diClBPA, 3,3′,5-triClBPA, and 3,3′,5,5′-tetraClBPA was detected at 30.6%, 4.8%, 4.8%, and 2.6% of the original BPA concentration after 5 minutes of reaction, and 57.8% of the original BPA still remained. No significant changes in the concentration of both the BPA and its chlorinated derivatives were observed throughout the experiment. On the other hand, trichlorophenols were not detected throughout the experiment.

From the above experiments, it was revealed that chlorine substitution number and formation of 2,4,6-triClPh also increased with the increasing chlorination time. In addition, it was also revealed from this experiment that when sodium hypochlorite higher than 1/5 of the original BPA concentration is added, formation of 3,3′-diClBPA was enhanced.

**Conclusions**

Studies were conducted to reveal the responses of Japanese medaka to various concentration ranges of BPA and chlorinated by-products of BPA. The conclusions drawn are as follows.

1. The estrogenic potencies of BPA and its chlorinated derivates were in the order of 3,3′-diClBPA>3-CIBPA>3,3′,5-triClBPA, and no estrogenic potency was observed for 3,3′,5,5′-tetraClBPA. The LOEC and NOEC were 500 µg/L and 200 µg/L, respectively, for both 3-CIBPA and 3,3′-diClBPA. LOEC for 3,3′,5-triClBPA was >500 µg/L.
2. When sodium hypochlorite was added to react with BPA, formation of 3-ClBPA, 3,3′-diClBPA, 3,3′,5-triClBPA, 3,3′,5,5′-tetraClBPA, and 2,4,6-triClPh was observed. The increase in chlorine substitution number was observed with the increase in reaction time. In addition, when sodium hypochlorite was added so that the residual chlorine was maintained at 1 ppm after 24 hours, not only the BPA but once formed chlorinated by-products also decomposed.

These findings present another problem as being a trade-off between the beneficial side of chlorination, which is a reduction in estrogenic activity or germicidal effect, and the disadvantageous side of chlorination, which is an increasing formation of chlorinated by-products such as chlorinated derivatives BPA and chlorophenol. Further study is necessary to identify the critical level of residual chlorine together with the minimum required reaction time for the elimination of the estrogenic activity of source water.

References


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