Bisphenol A Levels in Human Urine

Akiko Matsumoto,[1] Naoki Kunugita,[2] Kyoko Kitagawa,[1,] Toyohi Isse,[1] Tsunehiro Oyama,[1] Gary L. Foureman,[3] Masatoshi Morita,[4] and Toshihiro Kawamoto[1]*

[1]Department of Environmental Health and [2]School of Health Science, University of Occupational and Environmental Health, Kitakyusyu, Japan; [3]Hazardous Pollutant Assessment Group, National Center for Environmental Assessment, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA; [4]Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan

The estrogenic effects of bisphenol A (BPA) have been reported in human cells (E-screen assays) and in *in vivo* studies of rodents, although the latter reports remain controversial, as do the exposure levels and adverse health effects of BPA in humans. In this study we report on an analytical high-performance liquid chromatography/fluorescence method for BPA and its conjugate in human urine and on the application of this method in two student cohorts. Urine, along with information on smoking, alcohol intake, and coffee/tea consumption, was collected in two different years from two different groups of university students, 50 in 1992 and 56 in 1999. Overall, the urinary BPA levels in the students in 1992 were significantly higher than were those in 1999. The BPA levels were also positively correlated with coffee and tea consumption in the 1992 cohort but not in the 1999 cohort. We speculate that recent changes made in Japan regarding the interior coating of cans used to package these beverages may partly explain these findings. *Key words:* **biologic monitoring, bisphenol A, can coatings, canned food, environmental exposure, glucuronide, HPLC, human, lifestyle, urine.** *Environ Health Perspect* **111:101–104 (2003). [Online 31 October 2002]**
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Bisphenol A (BPA) is synthesized from acetone and phenol and is used mainly as an intermediate in the production of epoxy resins, polycarbonate resins, and polyester resins. Epoxy resins are applied in adhesives, coatings, plastics, and structural composites. Polycarbonates are used for a variety of plastic products for consumer use. In Japan, in 1999 the amount of BPA produced was estimated at 420,000 tons and the amount consumed at 405,000 tons, with demand increasing (*1*). At high concentrations, BPA was found to be estrogenic in MCF7 human breast cancer cells (E-screen assay), with the potency of the proliferative effect estimated to be about 10^{-4} to 10^{-6} times that of 17β-estradiol (*2,3*). Estrogenic effects have also been characterized in rodents. Recent studies report uterine and testicular effects among rats and mice exposed to BPA and prostate effects among mice having fetal exposure to BPA (*4–9*). In contrast, other recent experiments indicate few or no effects on reproductive function among rats administered BPA in the diet, although the BPA was relatively low (*10,11*).

Trace amounts of BPA are known to be eluted from polycarbonate plasticware and from resins used for food packaging (*12*), although actual human exposure via these sources remains to be confirmed and quantified.

In the present study we describe a simple method for the measurement of urinary BPA. We then applied this method to university students identified via a questionnaire regarding various behaviors and habits, including smoking and tea and coffee consumption—both beverages are readily available in cans coated with BPA-containing resins.

Materials and Methods

Chemicals. Bisphenol A [2,2-bis (4-hydroxyphenyl)propane], 1,1-bis(4-hydroxyphenyl)-ethane, acetonitrile, and tetrahydrofuran were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2,2-Bis(4-hydroxyphenyl)butane (bisphenol B) was obtained from Tokyo Chemical Industry Company (Tokyo, Japan). β-Glucuronidase/sulfatase (type H-2, from *Helix promatia*; β-glucuronidase activity, 110,000 U/mL; sulfatase activity, 4,000 U/mL) was from Sigma Chemical Co. (St. Louis, MO, USA).

Study subjects. Fifty university students (46 males, 4 females; mean age, 24.1 ± 2.2 years) were surveyed in 1992, and 56 (49 males, 7 females; mean age, 21.5 ± 1.3 years) in 1999. No randomization process was employed; participants were limited to the first 50 student volunteers. A morning spot urine specimen was collected from each student. All the collected urine specimens were kept at –80°C until analysis.

A questionnaire was administered to determine smoking habits/status (yes/no), diet [meat and fish (small/medium/large quantities), greasy foods (yes/no), highly seasoned foods (yes/no), sugary and fruits (yes/no)], alcohol intake (days/week), and coffee and/or tea (combined) consumption (amounts/day). The same questionnaire was used in both 1992 and 1999. Coffee and tea consumption was not separated in the questionnaire.

Analysis of urinary BPA. Urine (500 µL) was buffered with 30 µL of 2.0 M sodium acetate buffer (pH 5.0) and hydrolyzed enzymatically with β-glucuronidase/sulfatase (4,414/168 U/µL) for 3 hr at 37°C in a shaking water bath. After hydrolysis, 100 µL of 2N HCl was added, and the hydrolysate was extracted once with 5 mL of ethyl acetate with 10 µg/L bisphenol B (internal standard). After centrifugation, 4 mL of supernatant was transferred to a new tube and evaporated with N_2 gas. The residue was dissolved with 200 µL of 60% acetonitrile in water, and 40 µL of the solution was injected onto the high-performance liquid chromatography (HPLC) system described below. The total of conjugated plus unconjugated forms of BPA (total BPA) was measured by this procedure. The same procedure without β-glucuronidase/sulfatase was carried out in parallel to measure the unconjugated BPA (free BPA). The concentration of conjugated BPA was calculated by subtracting the amount of free BPA from the total BPA. The BPA concentration was also adjusted to the urinary creatinine concentration to correct the urine volume. The urinary creatinine concentration was determined by the method of Ogata and Taguchi (*13*), and the concentrations of free BPA, total BPA, and conjugated BPA are expressed in micrograms of BPA per gram of creatinine.

The HPLC system (L-7000 series; Hitachi High-Technologies Corporation, Tokyo, Japan) consisted of an L-7100 pump system operating at a 1.0 mL/min flow rate; the mobile phases were prepared by mixing acetonitrile, tetrahydrofuran, and water (35:35:130, 70:35:95) in the gradient mode; an L-7200 auto sampler, which injected 40 µL of the processed sample into the system; a Tosoh TSK gel ODS-80 column (6 mm inner diameter × 150 mm length). The system was equipped with a fluorescence detector (L-7400; Tosoh Corporation, Shin Nanyo, Japan).

Address correspondence to T. Kawamoto, Department of Environmental Health, University of Occupational and Environmental Health, Iseigaoka, Yahatanishi-ku, Kitakyusyu 807-8555, Japan. Telephone: 81-93-691-7243. Fax: 81-93-691-9341. E-mail: kawamott@med.uoeh-u.ac.jp

*Current address: 1st Department of Biochemistry, Hamamatsu University School of Medicine, Hamamatsu, Japan.

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Statistical methods. Nonparametric procedures, including the Mann-Whitney *U*-test and the Spearman rank correlation (r_s), were employed in statistical analysis of the data because of the sample size, variability of the data, and uncertainty about the underlying distribution.

Results

Measurements of BPA in urine. Representative chromatograms of the standard mixture, control urine spiked standard mixture, and student's urine are presented in Figure 1, showing 1,1-bis(4-hydroxyphenyl)ethane, BPA, and bisphenol B (internal standard) resolution by HPLC. The chromatogram of the spiked urine samples shows peaks at the same retention times as those of the standard mixture. Emission and excitation wavelength scans of the peak of a student's urine sample at the retention time of BPA (peak 2 in Figure 1C) were superimposable with those of BPA (Figure 2). The relationship between the fluorescence signal amplitude and the concentration of BPA from 5 µg/L to 100 µg/L was shown to be linear (Figure 3). The coefficient of variance and recovery rates for BPA were determined from spiking in urine and are shown in Table 1. From these data, we determined the limit of detection of this assay to be in the range of three times as much as standard deviation in control urine samples or around 1.7 µg/L urine (~7 nM).

Urinary BPA concentration of university students. Figure 4 shows that nearly all urinary BPA was present as conjugate (total minus free) in both sampling years. Although there was no significant difference in the mean levels of free BPA between the sampling years, the number of free BPA samples lower than the detection limit was higher in 1999 (50 of 56) than in 1992 (38 of 50). The median of total BPA in 1992 was significantly higher than that in 1999 by as much as 2.2-fold. Among the samples collected in 1992, urinary levels of both total and conjugated BPA (Figure 5) were higher in those students who consumed elevated amounts of coffee/tea (r_s = 0.297, $p < 0.05$). This trend was not observed among the students' urine samples collected in 1999 (r_s = –0.187, $p > 0.05$). This downward trend in the BPA levels from 1992 to 1999 was also reflected by the number of samples having nondetectable BPA levels (total)—that is, less than ~1.7 µg/L urine or 7 nM. In the 1992 cohort, only 18% (9 of 50) were nondetectable, whereas in the 1999 cohort this figure was increased to 39% (22 of 56). No or minimal relationships were shown in rank correlation of urinary BPA with smoking, alcohol intake, or dietary habits.

Data from male and female subjects were pooled together because no difference was reported in BPA metabolism between them in human subjects (*14*).

Discussion

Methods of BPA analysis. BPA levels in the environment (e.g., in water, food) have been successfully measured using HPLC and gas chromatography/mass spectrometry (*15–17*). Analyses for BPA in body fluids, such as

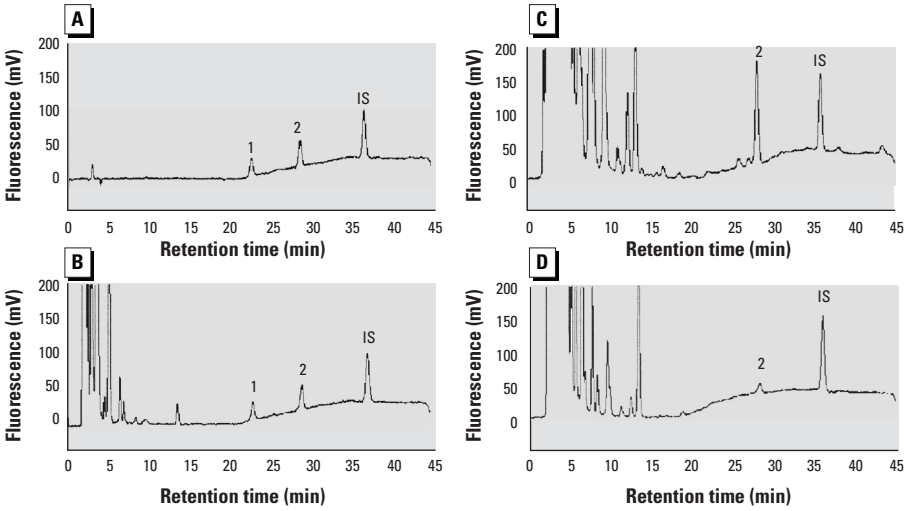

Figure 1. Chromatograms of standard mixture and urine samples. (*A*) Standard mixture of 1,1-bis(4-hydroxyphenyl)ethane (100 µg/L; peak 1), BPA (100 µg/L; peak 2), and bisphenol B (200 µg/L; peak IS, internal standard). (*B*) Control urine spiked with standard mixture [(1,1-bis(4-hydroxyphenyl)ethane, 100 µg/L, peak 1; BPA, 100 µg/L, peak 2; bisphenol B, 200 µg/L, peak IS]. (*C*) Student's urine with β-glucuronidase/sulfatase treatment (total). (*D*) Student's urine without β-glucuronidase/sulfatase treatment (free).

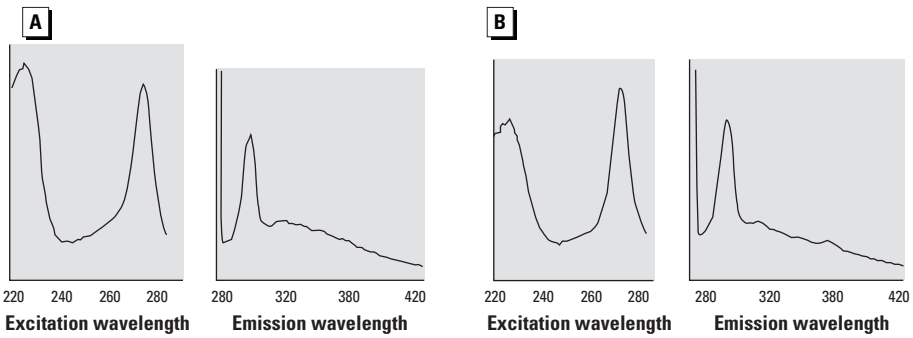

Figure 2. Emission and excitation wavelength scans for standard BPA and a hydrolyzed and extracted urine sample: emission wavelength scans by excitation at 275 nm and excitation wavelength scans by emission at 300 nm of (*A*) BPA standard and (*B*) peak 2 in Figure 1C.

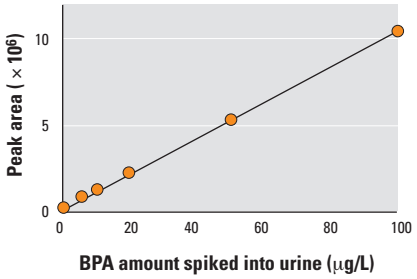

Figure 3. Relationship between BPA concentration spiked into urine sample and fluorescence signal amplitude. The urine was spiked with BPA (5–100 ng to 1 mL of urine) and a calibration curve was constructed from readings obtained with excitation at 275 nm and emission at 300 nm. The relationship is linear between 5 µg/L and 100 µg/L. $y = 1.00x + 0.263$; $r = 0.999$.

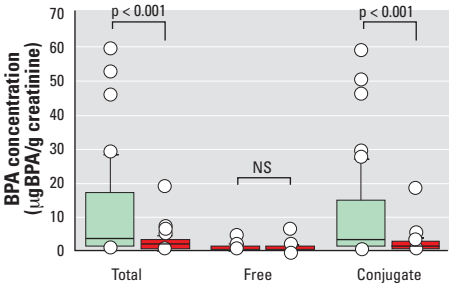

Figure 4. Comparison of BPA concentrations in the urine samples collected in 1992 and in 1999. NS, not significant. The upper and lower portions of the histogram for each category represent the 75th and 25th quartiles, respectively, and the lateral line within each histogram represents the median value. The lines extending above and below the histograms represent the 90th and 10th percentiles, respectively. Open circles indicate individual data from the 10–90th percentiles.

urine, however, are more difficult and require additional considerations not only because of matrix problems but also because of extensive metabolism of the parent compound. It has been established in rodents, for example, that BPA is extensively metabolized to glucuronides (57–98%) and possibly sulfate conjugates (0–4%), leaving 1–12% unmetabolized BPA (*18,19*), thereby complicating both qualitative and quantitative determinations. Another consideration, especially for urine sampling in workers, is contamination during the collection procedure.

The method presented in this report is simple and reliable and can be adapted to the assay of BPA glucuronide and sulfate conjugates that may occur in urine from the metabolism of BPA. For example, when BPA was orally administered to rats, 12–30% of the administered dose was excreted into their urine as the free form (< 1–12%), glucuronide (57% to >98%), and sulfate (0–4%) (*18,19*); in monkeys, 80–85% of the administered dose was excreted into urine, although the percentage of conjugates is unknown (*20*). Additionally, Dekant et al. (*14*) reported that orally dosed BPA (5 mg) was metabolized completely to glucuronide and excreted into urine within 24 hr in human subjects. Use of an enzymatic hydrolysis step as part of assays of glucuronides and sulfates has been demonstrated in a number of species and matrices, including bile and urine (*21,22*). The relatively high detection limit of the current version of this procedure, however, may limit its practical application for very low environmental levels of BPA, such as those recorded for the 1999 student cohort, in which more than half of the values were below 1.7 µg/L, the estimated limit of detection. Enzyme-linked immunosorbent assay and HPLC–electrochemical detector and HPLC–mass spectrometry methods have been developed (*23,24*) that could be adapted to our fluorescence method to extend the limit of detection downward. The recently published method of Brock et al. (*25*), who also assayed BPA in human urine, employs negative chemical ionization and selected ion monitoring in mass spectroscopic analysis to achieve a reported limit of detection of 0.12 ng/mL (0.12 µg/L).

BPA exposure in students. The urine samples collected in 1992 showed a clear trend between BPA levels and coffee and tea consumption, with a possible implication that a main source of the urinary BPA could be from the linings of cans containing these beverages. Obstacles in affirming this possibility are several and include the fact that the questionnaire in the present study addressed only coffee and tea, not canned coffee and tea. However, it is very popular to drink canned coffee and tea in Japan. For example, the market for canned coffee was 3.5×10^8 cases in 2001 (2.94×10^9 L/year), and the market for tea, including that sold in PET bottles, was just as high (*26*).

Elution of BPA from coatings in cans used for beverages has been confirmed to occur and is estimated at 0–213 ppb (0–213 ng/g) (*27–29*), with as much as 0–42 µg of BPA eluted from a typical 200 mL can. Using an assumed consumption rate of two cans of coffee each day (e.g., with 6 µg of BPA eluted/can), an assumed percentage of human excretion along with a creatinine correction factor of 1.2 g/day would yield urinary levels of 10 µg of BPA per gram of creatinine, within the range noted for urine from students collected in 1992. This BPA intake via canned coffee and tea is approximately 1/10,000 of the no observed adverse-effect level, for rats based on a three-generation reproductive toxicity study (*10*).

The trend of increasing urinary BPA levels as a function of coffee/tea consumption was not apparent in the urine samples collected in 1999. It is remotely possible that the low levels of BPA in these samples, which included many samples that had nondetectable levels of BPA, may have obscured the effect. Another reason for this lack of trend and the overall decrease in urinary BPA may be lower overall exposure to BPA, from canned beverages or otherwise. It should be noted that BPA contamination of canned beverages and foods became a matter of concern in Japan, and in 1997 most major manufacturing companies changed the interior can coatings to eliminate or reduce the use of BPA. An updated analysis of BPA elution from current can coatings may provides further support for this theory.

Table 1. Recovery and reproducibility of the BPA assay method.

Amount spiked (µg/L)	Average of amount detected (µg/L; $n = 5$)	Standard derivation	Coefficient of variance (%)	Recovery (%)
0	2.787	0.567	20.354	—
10	12.320	1.237	10.044	95.3335
20	21.397	1.286	6.011	93.0502
50	48.691	0.959	1.970	91.8073

Control urine samples (1 mL) were spiked with BPA at three levels, 10, 20, and 50 ng, and aliquots from each level were injected five separate times into the HPLC.

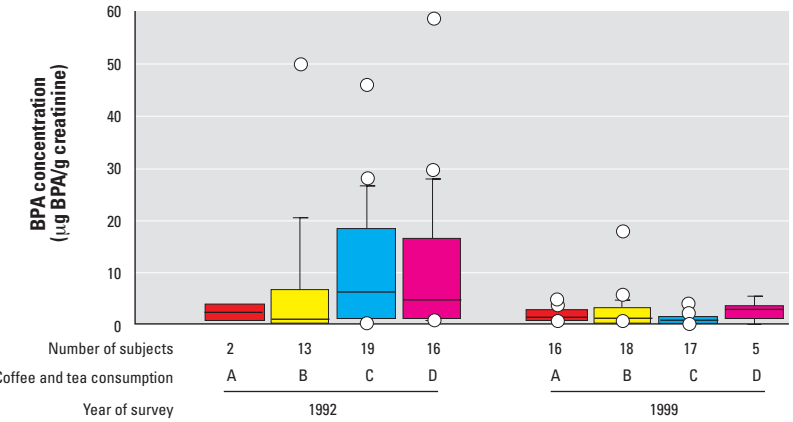

Figure 5. Urinary concentration of conjugated BPA in university students by coffee and tea consumption. The students were classified into four groups according to their coffee and tea consumption: (*A*) usually not taken; (*B*) 0–1 can or cup per day; (*C*) 1–2 cans or cups per day; (*D*) > 3 cans or cups per day. The upper and lower portions of the histogram for each category represent the 75th and 25th quartiles, respectively; the lateral line within each histogram represent the median value. The lines extending above and below the histograms represent the 90th and 10th percentiles, respectively. Open circles indicate individual data from the 10–90th percentiles. Rank-correlation coefficients (r_s) were computed comparing individual urinary BPA levels with these four categories.

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