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Gender differences in the levels of bisphenol A metabolites in urine

Yong-Hak Kim,[a] Cheong-Sik Kim,[a] Soyeon Park,[a] Soon Young Han,[b] Myoung-Yun Pyo,[c] and Mihi Yang[a,d,*]

[a] *Department of Preventive Medicine, School of Medicine, Seoul-National University, 28 Yon-gun Dong, Chong-no Gu, Seoul 110-799, Republic of Korea*
[b] *Endocrine Toxicology Division, National Institute of Toxicological Research, Korea Food and Drug Administration, 5 Nokbun-dong, Eunpyung-ku, Seoul 122-704, Republic of Korea*
[c] *Department of Pharmacy, Sookmyung Women's University, Chungpa-Dong, Yongsan-Ku, Seoul 140-742, Republic of Korea*
[d] *Cancer Research Institute, School of Medicine, Seoul-National University, 28 Yon-gun Dong, Chong-no Gu, Seoul 110-799, Republic of Korea*

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Abstract

The metabolism of bisphenol A (BPA), a suspected endocrine disruptor, should be considered for monitoring human exposure to BPA, because the conjugation with β-D-glucuronide and sulfate reduces the estrogenic activity. In this study, BPA levels in 30 healthy Koreans (men, $N = 15$, 42.6 ± 2.4 years; women, $N = 15$, 43.0 ± 2.7 years) were analyzed from urine treated with/without β-glucuronidase and/or sulfatase by an RP-HPLC with fluorescence detection. The total BPA concentrations including free BPA and the urinary conjugates were similar in men and women (2.82 ± 0.73 and 2.76 ± 0.54 ng ml^{-1}, respectively), but gender differences were found in the levels of urinary BPA conjugates. Men had significantly higher levels of BPA–glucuronide (2.34 ± 0.85 ng ml^{-1}) than women (1.00 ± 0.34 ng ml^{-1}), whereas women had higher levels of BPA–sulfate (1.20 ± 0.32 ng ml^{-1}) than men (0.49 ± 0.27 ng ml^{-1}).

Keywords: Bisphenol A; Endocrine disruptor; β-Glucuronidase; Sulfatase; Urinary conjugates

Environmental contaminations by endocrine disruptors (EDs) have been suggested to be a cause of human health disorders. Although no clear evidence has been presented or mechanism proposed for the effects of EDs on humans, many studies on experimental animals and wild lives have emphasized the potential threat to human health posed by chronic exposure to EDs [1–5].

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane), a suspected ED, is widely used in the synthesis of polycarbonate plastics, epoxy resins, and composites. The metabolic products with hydrophilic substituents are likely excluded through urine excretes. Particularly, the conjugation with β-D-glucuronide, which is commonly found in rat and human liver microsomes, appears to have a considerable effect on lowering the estrogenic activity of BPA in vivo [6]. BPA sulfate is rarely found in humans, but it seems to have a similar effect with BPA–glucuronide in rat hepatocytes. The BPA conjugates, which are excreted to the dietary tract, can resorb via the microbial degradation to free BPA in the colon. In addition, the phenolic moieties of BPA efficiently trap free radicals [7], and it is oxidized by radical oxidants, Fremy's salt, tyrosinase [8], and lipoperoxides which are formed by the auto-oxidation of lipids in the presence of reactive oxygen species [9]. BPA is also converted to 5-hydroxybisphenol A by rat and human liver microsomes in the presence of NADPH, but when this pathway is assessed for the impact on the estrogenicity in the coupled yeast assay, it has no significant effect on lowering the estrogenic activity of BPA due to a low metabolic turnover in microsomal system [6]. The oxidative BPA metabolism may only occur in vivo at high doses following the saturation of other metabolic pathways [10,11]. Accordingly, the metabolic turnovers for the BPA oxidation are very low to have a

* Corresponding author. Fax: +82-2-747-4830.
E-mail address: myang@snu.ac.kr (M. Yang).

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significant effect on lowering the estrogenic activity [6,10].

To a certain degree, the BPA metabolism to produce the conjugates with β-D-glucuronide and sulfate may have effects on lowering the estrogenic activity of BPA, and plays an important role in eliminating the risks through urine excretion from body, especially tissues targeted by estrogen. To properly monitor human exposure to BPA using urine samples, the BPA metabolism should be considered. This study presents gender differences in the levels of urinary BPA–glucuronide and –sulfate in normal Koreans. To accurately and precisely determine the total BPA concentrations including non-metabolized free form of BPA, BPA–glucuronide and –sulfate, BPA was analyzed by a reversed phase HPLC with fluorescence detection (HPLC/FD), after 1 h of the incubation of urine in the absence or presence of β-glucuronidase [EC 3.2.1.31] and/or sulfatase [EC 3.1.6.1].

Materials and methods

Chemicals and reagents. BPA and 4-nitrophenyl sulfate (potassium salt) were purchased from Aldrich, (Milwaukee, WI). Bisphenol B (BPB) was supplied by Tokyo Kasei Kogyo (Tokyo, Japan). Dithiothreitol (DTT), β-glucuronidase from *Escherichia coli* (*E. coli*), 1-naphthyl-β-D-glucuronide, 1-naphthyl sulfate, 4-nitrophenyl-β-D-glucuronide, TRIZMA, and TRIZMA-HCl were purchased from Sigma (St. Louis, MO). A β-glucuronidase mixture of *Helix pomatia* (*H. pomatia*) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). All other solvents and chemicals used in sample preparations and HPLC buffers were of the HPLC grade, J.T. Bakers (Philipsburg, NJ), and water was deionized using a Millipore Super-Q Plus water purification system (Bedford, MA).

Enzyme assays. For the β-glucuronidase assay, 1-naphthyl-β-D-glucuronide and 4-nitrophenyl-β-D-glucuronide were used as chromogenic substrates, and the sulfatase activity was assayed with 1-naphthyl sulfate or 4-nitrophenyl sulfate. One milliliter of the reaction mixture consisted of 20 μl of enzyme solution, 20 μl of 10 mM of a chromogenic substrate dissolved in water, and 960 μl of 50 mM Tris–HCl buffer (pH 6.7). The reaction was started by adding a chromogenic substrate.

Enzyme activity was determined at 25 °C for 10 s with measuring the increase of absorbance of 1-naphthol at 323 nm ($\varepsilon = 1680\,M^{-1}\,cm^{-1}$) or 4-nitrophenol at 405 nm ($\varepsilon = 1850\,M^{-1}\,cm^{-1}$). One unit (U) of enzyme activity was defined as the production of 1 μmol of 1-naphthol or 4-nitrophenol per min. Protein concentrations were determined by a Bio-Rad assay kit using bovine serum albumin as a standard.

Optimization for enzyme treatments. The activities of β-glucuronidase and sulfatase were measured for 10 s at 25 °C under various pH conditions to investigate their pH-dependences. The pH of buffers varied with 100 mM sodium citrate–HCl (pH 4.0–6.8) and 50 mM Tris–HCl (pH 6.7–9.0). At the optimal pH conditions, the thermal denaturation of proteins was examined by measurements of the enzyme activity at 2–10 min intervals during the incubation at 15–95 °C, and protein denaturation constants, k_d (min^{-1}), were determined by the simple first-order kinetic equation, $U_t = U_0 \times \exp(-k_d \times t)$, in which U_0 and U_t are enzyme activities at time 0 and t.

Enzyme purification. In order to avoid signal interferences raised from contaminants in the commercial β-glucuronidase and sulfatase mixtures, the enzymes were purified by a high performance gel filtration. A narrow bore high performance column (3.9 × 300 mm) was packed with Superose12 (particle size, 10 μm, Pharmacia Biotech., Uppsala, Sweden) suspended in 50 mM Tris–HCl (pH 6.7). After the equilibration with 50 mM Tris–HCl (pH 6.7) and 1 mM DTT at a flow rate of 0.1 ml min^{-1}, 20 μl of a crude enzyme extract containing 1–2.5 mg of protein was loaded, and the protein elution was monitored at 280 nm with 200 μl fractions being collected. Aliquots (20 μl) of the fractions were assayed for the enzyme activities towards 4-nitrophenyl-β-D-glucuronide and -sulfate at a final concentration of 200 μM. The molecular mass of native protein was determined using blue dextran (2000 kDa), alcohol dehydrogenase (200 kDa), β-amylase (150 kDa), bovine serum album (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa), as gel filtration molecular weight markers (Sigma, St. Louis, MO).

Preparation of calibration curves. A standard curve (STD_{BPA}) was prepared using five concentrations of BPA in a range of 2–20 ng ml^{-1}. To prepare the calibration curves ($STD_{extract}$), the same concentrations of BPA spiked with 50 mM Tris–HCl buffer (pH 6.7) or urine were treated in the same manner as described below in the preparation of urine samples. The efficiency of the *tert*-butyl methyl ether (TBME) extraction was calculated from the percent ratio of the slope of ($f_i \times STD_{extract}$) to that of STD_{BPA}, where f_i is the sample dilution factor for i, individual standards and samples treated or not treated with enzyme(s).

Preparation of urine samples. Urine samples were obtained from 30 Korean volunteers (men, $N = 15$: age, 42.6 ± 2.4 years; women, $N = 15$: age, 43.0 ± 2.7 years) in Daejon, central South Korea, and frozen at −20 °C until used. The volunteers were ranked as normal adults who had experienced neither occupational exposure to BPA nor endocrine disorder. There was no user of oral contraceptives among the female subjects.

For the preparation of standards and samples, only glassware was used to avoid BPA contamination by plastic goods. A half milliliter of urine was treated with 12.5 U of β-glucuronidase (BPA_G) or a mixture of 12.5 U of β-glucuronidase and 1.25 U of sulfatase (BPA_T) at 35 °C for 1 or 3 h, but the incubation periods for 1 and 3 h had no significant difference in total BPA concentrations (data not shown). The enzyme reactions were stopped by adding 2.5 ml of ice-cold TBME containing 10 ng ml^{-1} bisphenol B (BPB) as an internal standard. The reaction mixture was then vigorously mixed and frozen at −20 °C for 30 min to separate aqueous and TBME phases. Aliquots (2 ml) of the TBME extracts were then transferred to clean test tubes and dried in vacuo using a Speed-Vac system (Savant Instruments, Farmingdale, NY). To determine concentrations of non-metabolized free BPA (BPA_F), urine samples without addition of an enzyme were included under the same conditions. If urinary conjugates were completely degraded by the two enzymes, the total concentration of metabolized BPA was determined from the difference between BPA_T and BPA_F. The concentration of BPA–glucuronide was difference between BPA_G and BPA_F, and the concentration of BPA–sulfate was difference between BPA_T and BPA_G. Because of a possible existence of an enzyme inhibitor in urine, 200 μM of each of 4-nitrophenyl-β-D-glucuronide and -sulfate was also included as substrate analogues under the same conditions to calculate the efficiency coefficients of β-glucuronidase (k_{GUS}) and sulfatase (k_{ARS}). Using the efficiency coefficients, the total BPA concentration, BPA_{total}, was calculated as follows:

$$BPA_{total} = (f_F \times BPA_F) + k_{GUS} \cdot \{(f_G \times BPA_G) - (f_F \times BPA_F)\} + k_{ARS} \cdot \{(f_T \times BPA_T) - (f_G \times BPA_G)\}$$

in which f_F, f_G, and f_T were sample dilution factors for every measurement for BPA_F, BPA_G, and BPA_T, respectively.

Analytical method. BPA levels were analyzed by HPLC using a Waters 474 Scanning Fluorescence detector (HPLC/FD) set at 275 nm for excitation and 300 nm for emission with a slit width of 10 nm [12]. The chromatographic system consisted of dual Waters 515 HPLC

pumps, a Waters Automated Gradient Controller (Waters, Milford, MA), and a Symmetry C18 column, 5 μm, 4.6 × 250 mm (Waters, Ireland). One hundred microliters of samples was consecutively injected using a Waters 717 plus Autosampler installed with a 250 μl loop. A linear gradient of mobile phase was applied for 30 min from 100% buffer A (10%/90% (v/v) acetonitrile/water + 0.1 mM octanesulfonic acid) to 70% buffer B (90%/10% (v/v) acetonitrile/water + 0.1 mM octanesulfonic acid), pumped at 1 ml min^{-1}. The column was then washed with 100% buffer B for 10 min and reconstituted with 100% buffer A for 10 min. One cycle of the HPLC analysis took 52 min, inclusive of the next sample injection delay of 2 min. In the preliminary studies, the use of a constant octanesulfonic acid (sodium salt) at 0.1–1 mM in the mobile phase was proper to have biological components in urine early eluted by ion-pairing forces, resulting in the clear detection of the peaks of BPA and BPB by this method.

4-Nitrophenyl derivatives and the enzyme reaction product, 4-nitrophenol, were detected using a Waters 486 Tunable Absorbance detector at 254 nm. The chromatographic system was same as above mentioned. A linear gradient of mobile phase, pumped at 1 ml min^{-1}, was applied for 30 min from 10%/90% (v/v) acetonitrile/water to 70%/30% (v/v).

To validate the HPLC/FD method, gas chromatography/electron ionization mass spectrometry (GC/EI-MS) was performed using an Agilent 6890 Gas Chromatography (Agilent Technologies, Palo Alto, CA) equipped with an Autoliquid Sampler and an Agilent 5973N Mass Selective Detector. BPA and BPB extracted from 1 ml urine samples treated with β-glucuronidase and sulfatase as described above were derivatized with 50 μl of bis(trimethylsilyl)trifluoroacetamide in the presence of dibromophenol as a surrogate and dibromotoluene as an internal standard. The analytical column used was a DB-5MS column, 30 m × 0.25 mm I.D., 0.25 μm (Agilent Technologies, Palo Alto, CA), flowed with He gas at 1.0 ml min^{-1}. The split ratio of the inlet was 10:1. Temperatures of the injector and the detector were 270 and 280 °C, respectively, and the electron energy was 70 V. The oven temperature was increased at 2 min from 50 to 280 °C at 10 °C min^{-1} and held at 280 °C for 5 min.

Statistics. The Wilcoxon rank-sum test was used to study significant differences in the concentrations of various BPA forms between the two groups (SAS software, version 8.1, SAS Institute, Cary, NC).

Results

Enzyme purifications

A commercial β-glucuronidase mixture from *H. pomatia* had a low activity for the hydrolysis of 4-nitrophenyl-β-D-glucuronide (Fig. 1A). In contrast, this commercial enzyme mixture included a strong sulfatase activity for hydrolyzing 4-nitrophenyl sulfate and the native protein had the molecular mass of 101 kDa. Purification of the sulfatase by gel filtration was contaminated with a quantity of β-glucuronidase which had the molecular mass of 113 kDa. The gel filtration enabled the purification of β-glucuronidase and sulfatase from the commercial enzyme mixture with yields of 40% and 48%, respectively, and their specific activities increased by 3- to 4-fold.

Another commercial mixture from *E. coli* contained a high activity of β-glucuronidase, but it had no sulfatase activity (Fig. 1B). The β-glucuronidase was eluted from gel filtration at 114 kDa and appeared to be a homodimer. The gel filtration enabled the purification of the β-glucuronidase with yield of 28% and the specific activity increased by 2.7-fold.

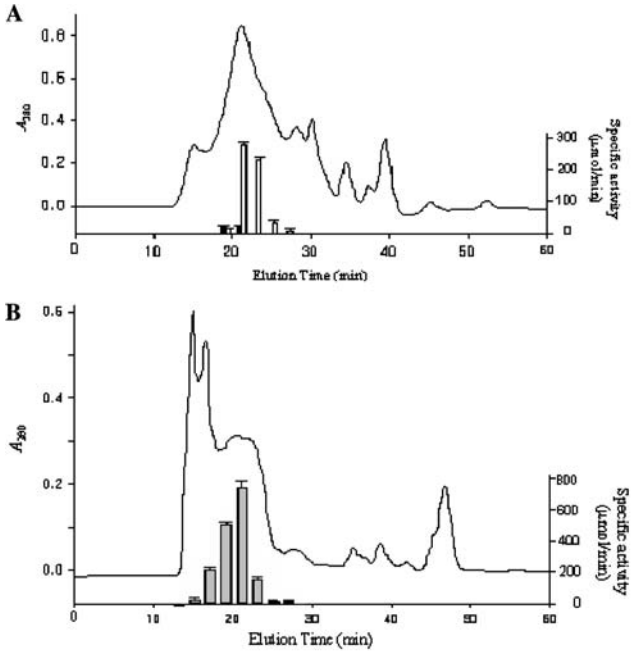

Fig. 1. (A) Gel chromatogram of a commercial β-glucuronidase of *H. pomatia* (555 μg protein). The respective activities of β-glucuronidase and sulfatase towards 4-nitrophenyl-β-D-glucuronide and -sulfate are shown with black and white bars. (B) Gel chromatogram of a commercial β-glucuronidase of *E. coli* (260 μg protein). The β-glucuronidase activity towards 4-nitrophenyl-β-D-glucuronide is shown with gray bars.

Substrate specificity

Substrate specificities of purified β-glucuronidases and sulfatase were examined with 4-nitrophenyl and 1-naphthyl derivatives (Table 1). The β-glucuronidase from *H. pomatia* was able to hydrolyze 4-nitrophenyl-β-D-glucuronide, but not 1-naphthyl-β-D-glucuronide. In contrast, another β-glucuronidase from *E. coli* was able to hydrolyze 4-nitrophenyl-β-D-glucuronide and 1-naphthyl-β-D-glucuronide. The β-glucuronidase of *E. coli* appeared to have the specificity for a broad range of phenyl- and naphthyl-β-D-glucuronides.

The sulfatase from *H. pomatia* showed a high activity towards 4-nitrophenyl sulfate, but not towards 1-naphthyl sulfate.

Effects of pH and temperature on enzyme activity

To optimize the enzyme reactions with urinary BPA conjugates, it was necessary to investigate the pH-dependences and thermodynamic behaviors of the enzymes. The catalytic activity of the β-glucuronidase from *E. coli* towards 200 μM 4-nitrophenyl-β-D-glucuronide

Table 1
Purification of β-glucuronidase and sulfatase by gel filtration

Origin	Enzyme	Substrate[a]	Specific activity (U mg^{-1} protein)	
			Commercial products	Gel filtration
Helix pomatia	β-Glucuronidase	1-NaG	ND[b]	ND
		4-NPG	87.7 (2.92)	259.5 (86.5)
	Sulfatase	1-NaS	ND	ND
		4-NPS	1061 (77.5)	3805 (173)
Escherichia coli	β-Glucuronidase	1-NaG	1782 (60.6)	5031 (284)
		4-NPG	10,053 (10.4)	27,818 (1209)
	Sulfatase	1-NaS	ND	ND
		4-NPS	ND	ND

[a] Substrate (final concentration, 200 μM): 1-NaG, 1-naphthyl-β-D-glucuronide; 4-NPG, 4-nitrophenyl-β-D-glucuronide; 1-NaS, 1-naphthyl sulfate; 4-NPS, 4-nitrophenyl sulfate (potassium salt).
[b] ND, not detectable.

was stable in 50 mM Tris–HCl (pH 6.7) at below 45 °C, but the activity was rapidly lost at above 55 °C due to thermal denaturation of the protein (Fig. 2A).

The sulfatase from *H. pomatia* displayed an excellent stability against temperature at below 55 °C (Fig. 2B). Even a heat treatment at below 55 °C appeared to stimulate the sulfatase activity. This may be due to the fact that the heat treatment resulted in a conformational change to increase the sulfatase activity. At above 65 °C, the sulfatase lost the activity rapidly (Fig. 2B).

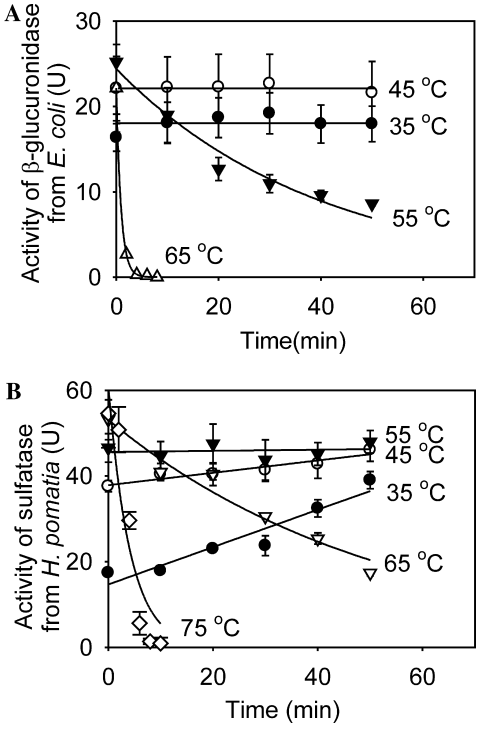

Fig. 2. Time-coursed activity assays for (A) β-glucuronidase from *E. coli* (0.5 μg ml^{-1}) towards 200 μM 4-nitrophenyl-β-D-glucuronide and (B) sulfatase from *H. pomatia* (5 μg ml^{-1}) towards 200 μM 4-nitrophenyl sulfate in 50 mM Tris–HCl (pH 6.7) at various temperatures.

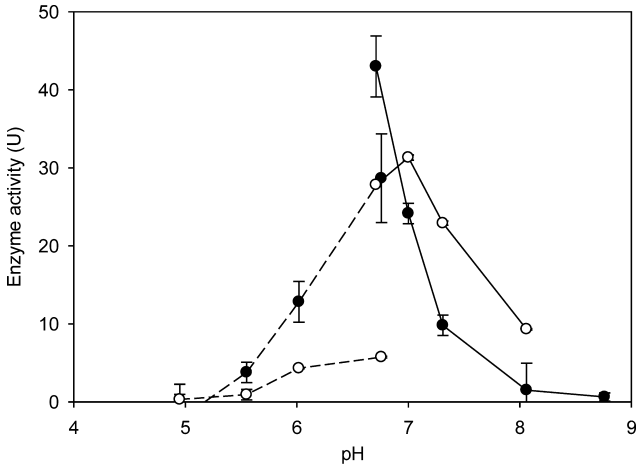

Fig. 3. The pH-dependences of β-glucuronidase from *E. coli* (○, 1 μg ml^{-1}) for 4-nitrophenyl-β-D-glucuronide (200 μM) and sulfatase from *H. pomatia* (●, 10 μg ml^{-1}) for 4-nitrophenyl sulfate (200 μM) at 25 °C. The pH of buffers varies with 100 mM sodium citrate–HCl (dashed lines; pH 4.0–6.8) and 50 mM Tris–HCl (bold lines; pH 6.7–9.0).

The uses of β-glucuronidase from *E. coli* and sulfatase from *H. pomatia* appeared to be suitable for the hydrolysis of phenol conjugates with β-D-glucuronide and sulfate in the physiological pH range (Fig. 3). When β-glucuronidase (25 U ml^{-1}) and sulfatase (2.5 U ml^{-1}) were directly used in urine samples without a pH buffer, 99.6% of the 4-nitrophenyl-β-D-glucuronide and 96.3% of the 4-nitrophenyl sulfate were converted to 4-nitrophenol within 1 h at 35 °C.

Determination of BPA in urine samples by HPLC/FD

By a reversed phase HPLC with fluorescence detection (HPLC/FD), BPA was detected to 0.28 ng ml^{-1} at a signal/noise ratio of 3. As seen in Fig. 4, the peaks of BPA and BPB were reproducibly detected at 22.6 and 25.1 min, respectively.

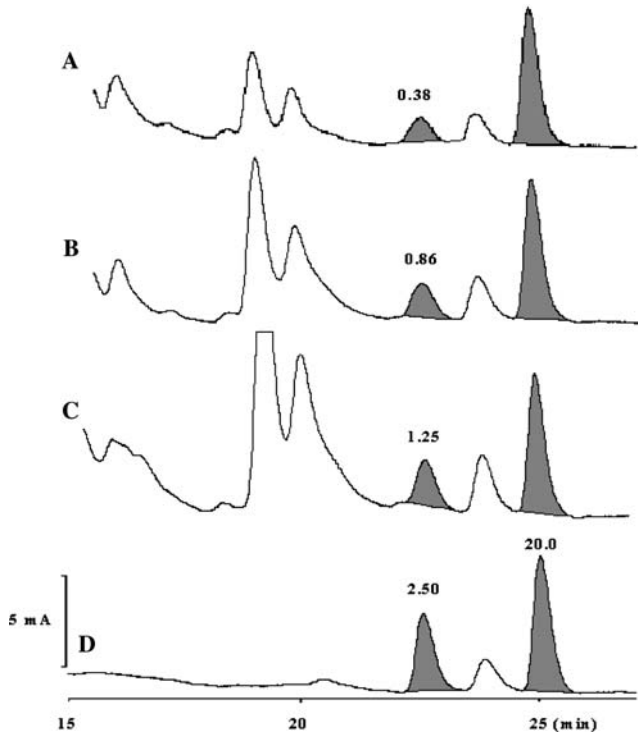

Fig. 4. Typical chromatograms for a urine sample (A) not-treated with enzyme, and (B) treated with β-glucuronidase (25 U ml^{-1}) and (C) β-glucuronidase (25 U ml^{-1}) + sulfatase (2.5 U ml^{-1}), and (D) a standard including BPA (2.5 ng ml^{-1}) and BPB (20 ng ml^{-1}). The determined concentrations of BPA and BPB are seen above the respective shaded peaks at 22.6 and 25.1 min.

For the determination of concentrations of free form of BPA and the major metabolites including BPA–glucuronide and BPA–sulfate, several factors were considered to correct the measured data. The direct use of a commercial enzyme mixture made it difficult for identification of the BPA peak interfering with the exogenous contaminants. Instead, the use of purified enzymes was adequate for eliminating the signal interferences. BPA spiked with urine (range of 2–20 ng ml^{-1}) displayed a good linearity ($r > 0.99$).

BPA concentration in sample, i, was multiplied by the factor, f. The f value was an inverse ratio of the BPB concentration of the sample (BPB_{Xi}) to the mean value of standards ($BPB_{\bar{X}}$), i.e., $f = BPB_{\bar{X}}/BPB_{Xi}$. From correction of the measured calibration curves with individual f values, BPA extraction with TBME resulted in the efficiency of almost 100% (Table 2).

The efficiency of urine treatments with β-glucuronidase and/or sulfatase seemed to be related to the catalytic properties and specificity of the enzyme(s). Since these hydrolases appeared to have high specificities for *p*-substituted phenol conjugates with β-D-glucuronide and sulfate, the efficiency coefficients were calculated from the enzyme reactions with structurally similar substrates in excess. β-Glucuronidase from *E. coli* (25 U ml^{-1}) was capable of hydrolyzing more than 99% of 200 μM 4-nitrophenyl-β-D-glucuronide at 35 °C for 1 h ($k_{GUS} = 0.996 \pm 0.003$), and sulfatase from *H. pomatia* (2.5 U ml^{-1}) hydrolyzed about 96% of 200 μM 4-nitrophenyl sulfate at 35 °C for 1 h ($k_{ARS} = 0.963 \pm 0.015$). Accordingly, it was assumed that these enzymes were able to almost completely hydrolyze BPA–glucuronide and –sulfate much less than the substrate-analogous 4-nitrophenyl derivatives.

With correction of the measured data by the above-mentioned factors, concentrations of BPA, BPA–glucuronide, and –sulfate were determined from urine samples (Table 3). Levels of total and free BPA were similar in men and women. The ratios of free BPA to total BPA were also similar in men and women. However, the levels of BPA–glucuronide and –sulfate were significantly different between the two sexes ($p < 0.01$). BPA–glucuronide was the major urinary conjugate in men, whereas the levels of BPA–glucuronides and –sulfates were similar in women. This result indicated that women had a greater ability for the sulfation of BPA than men.

Table 2
Efficiency of *tert*-butyl methyl ether extraction of bisphenol A

Samples (0–20 ng ml^{-1})[a]	Linearity[b]: $y = ax + b$			% Recovery[c]	
	a (SE)	b (SE)	R^2	Measured	Corrected
Standard curve	5.38 (0.054)	0.81 (0.60)	0.9998	100	100
Calibration curves					
No enzyme	5.91 (0.063)	1.39 (0.71)	0.9998	109.9	103.3
GUS[d]	7.19 (0.047)	7.77 (0.53)	0.9999	133.6	102.4
GUS + ARS[e]	6.99 (0.18)	5.21 (1.99)	0.9987	129.8	109.9

[a] BPA was treated at five concentrations in the range 0–20 ng ml^{-1}.
[b] The curve linearity was calculated by the least square method with measured data for BPA.
[c] Recovery was calculated from the percent ratio of the slope (a) of calibration curve, measured after 1 h of treatment with or without enzyme(s), to that of standard curve. BPA concentrations of individuals were corrected by multiplication of the corresponding sample dilution factors, f, which were accounted for the ratios of the average BPB peak area of the standards to individual BPB peak areas of samples, i.e., $f = BPB_{\bar{X}}/BPB_{Xi}$.
[d] GUS, β-glucuronidase from *E. coli* (25 U ml^{-1}).
[e] ARS, sulfatase from *H. Pomatia* (2.5 U ml^{-1}).

Table 3
Comparison of urinary BPA levels between male and female subjects

Sex	Types of BPA	Range	Mean (SE)	% (SE)
Men	Free BPA	0.28–2.36	0.58 (0.14)	29.1 (5.04)
	BPA–glucuronide	0.16–11.67	2.34 (0.85)**	66.2 (8.41)
	BPA–sulfate	<MDL[a]–1.03	0.49 (0.27)**	4.78 (7.77)
	Total	0.85–9.83	2.82 (0.73)	100
Women	Free BPA	0.068–1.65	0.56 (0.10)	33.4 (7.41)
	BPA–glucuronide	<MDL[a]–4.34	1.00 (0.34)**	33.1 (6.43)
	BPA–sulfate	<MDL–3.40	1.20 (0.32)**	33.5 (8.40)
	Total	1.00–7.64	2.76 (0.54)	100

[a] <MDL, below minimum detection limit.
** $p < 0.01$, compared between the two sexual groups.

Validation tests

Among five samples randomly selected from 30 urine samples, a significant correlation ($r > 0.998$) was confirmed between the total BPA levels obtained from the above-mentioned HPLC/FD analysis and the GC/EI-mass analysis (Fig. 5). There was no statistically significant difference ($P > 0.05$) in the total BPA concentrations determined by the two analytical methods. By the GC/EI-mass analyses, the standard curves for BPA and BPB resulted in a high linearity with six concentrations in a range of 1–500 ng ml^{-1} ($R^2 > 0.999$), but the minimum detection limit of 1 ng ml^{-1} was greater than that resulting from the HPLC/FD method (0.28 ng ml^{-1}). The mass spectrum of BPA detected at 21.0 min showed the molecular ion of m/z 372 [$M^{+\cdot}$] and the base peak at m/z 357 [$M^{+\cdot}$–CH_3], and the mass spectrum of BPB detected at 21.7 min showed the molecular ion of m/z 386 [$M^{+\cdot}$] and the base peak at m/z 357 [$M^{+\cdot}$–CH_2CH_3]. Thus, BPA and BPB in urine extracts were quantified by the selective ion mode (SIM) with the respective molecular ions and the base peak at m/z 357. Efficiency of the trimethylsilyl derivatization of samples was calculated with the recovery of dibromophenol detected with the selective ion of m/z 309 [$M^{+\cdot}$–CH_3] at 14.2 min. Artifacts raised from the validation tests were corrected by the detection of dibromotoluene with the selective ions of m/z 250 [$M^{+\cdot}$] and 169 [$M^{+\cdot}$–HBr] at 11.1 min.

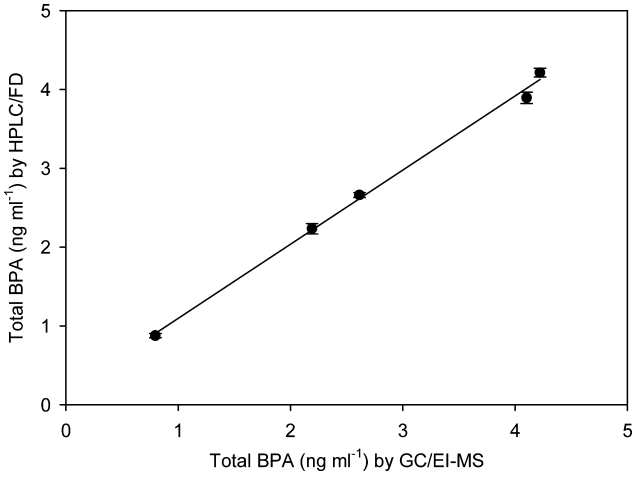

Fig. 5. Comparison of reversed phase high performance liquid chromatography with fluorescence detection (HPLC/FD) and gas chromatography/electron ionization mass spectrometry (GC/EI-MS) for the determination of total BPA concentrations.

The BPA metabolism curves

To understand the risk response of body to BPA exposure, plots of the percentage of metabolized BPA vs. total BPA concentration were fitted to the two-parameter exponential equation:

$$\%\ \mathrm{BPA}_{\mathrm{metabolized}} = \%\ \mathrm{BPA}_{\mathrm{max}} \times \left(1 - \mathrm{e}^{-k[\mathrm{BPA}_{\mathrm{total}}]}\right), \qquad (1)$$

where % $\mathrm{BPA}_{\mathrm{max}}$ is the maximum percentage of BPA metabolized, and the minus sign of the constant k indicates the catalytic activity for the BPA conjugation with β-D-glucuronide and sulfate. However, it is difficult to define the overall catalytic reactions for BPA glucuronidation and sulfation by the simple first-order kinetics. Therefore, it may be more meaningful to express the plots in terms of the dimensionless coefficient, a ($0 < a < 1$), in order to determine the dose–response relation to the total BPA concentration:

$$\%\ \mathrm{BPA}_{\mathrm{metabolized}} = \%\ \mathrm{BPA}_{\mathrm{max}} \times \left(1 - a^{[\mathrm{BPA}_{\mathrm{total}}]}\right). \qquad (2)$$

This equation implies that the BPA metabolism is triggered more sensitively with a small change in the total BPA concentration, as the response-coefficient (a) is smaller. The greater the % $\mathrm{BPA}_{\mathrm{max}}$ value is, the greater the catalytic activity is at high doses. As shown in Fig. 6, the measured data points closely corresponded with the model equation. The asymptotic (% $\mathrm{BPA}_{\mathrm{max}}$) values were estimated to be $88.9 \pm 8.0\%$ for men and $98.4 \pm 15.5\%$ for women, and the respective response-coefficients, a, were 0.386 ± 0.094 and 0.578 ± 0.115. Accordingly, men appeared to respond more sensitively to BPA exposure than women.

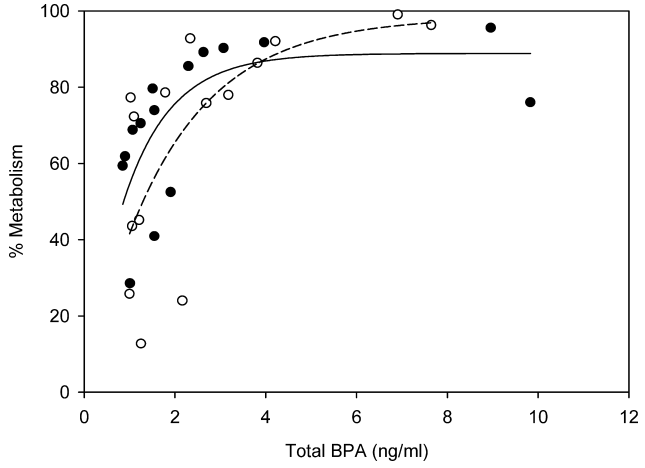

Fig. 6. Plots of the percentage of BPA metabolized vs. total BPA concentration (●, men; ○, women). The bold and dashed lines are generated from the curve fittings of the data points to the two-parameter exponential Eq. (2) for men and women, respectively.

Discussion

Human exposure to environmental estrogen-like chemical BPA, if not overcome, will add extra estrogenic burden to the system at elevated doses [13]. Elsby et al. [6] reported that the estrogenic activity of BPA was reduced 3- and 7-fold by the glucuronidation in women and immature female rats, respectively. They suggested that tissues targeted by estrogen in humans may be subjected to greater exposure to BPA than those in immature female rats, since human liver microsomes do not glucuronidate BPA so extensively as rat liver microsomes. Volkel et al. [14] suggested that glucuronidation of BPA and the rapid excretion are more effective in lowering the estrogenic activity of BPA in humans rather than in rats. Therefore, there are strain-related differences in the BPA metabolism, which may influence the susceptibility to the BPA toxicity. To more or lesser extent, BPA conjugation with β-D-glucuronide and sulfate may be the representative mechanisms for lowering the estrogenic activity of BPA in mammalian systems.

A commercial β-glucuronidase/sulfatase mixture from *H. pomatia* has been widely used to hydrolyze the urinary conjugates of steroids [15,16] and isoflavones [17]. It seems to provide a convenient means for the determination of total concentrations of the urinary conjugates. However, it cannot be used for determining the levels of individual BPA conjugates with β-D-glucuronide and sulfate. On the other hand, a commercial β-glucuronidase from *E. coli* includes only β-glucuronidase without sulfatase activity (Table 1). The β-glucuronidase from *E. coli* has the specificity for a broad range of phenyl- and naphthyl-β-D-glucuronides. Thus, the use of β-glucuronidase purified from *E. coli* alone or in combination with sulfatase purified from *H. pomatia* seems to be more practical for analyzing the levels of individual BPA conjugates in urine.

Takeuchi and Tsutsumi [18] found that free BPA concentrations in serum samples obtained from normal men ($1.49 \pm 0.11\,\mathrm{ng\,ml^{-1}}$) and women with polycystic ovary syndrome ($1.04 \pm 0.10\,\mathrm{ng\,ml^{-1}}$) were significantly higher than those from normal women ($0.64 \pm 0.10\,\mathrm{ng\,ml^{-1}}$), and that the serum BPA levels correlated positively to free and total testosterone concentrations in all subjects. They suggested that there are gender differences in serum BPA concentrations, possibly due to the difference in androgen-related enzyme activity for the BPA metabolism. In this study, no gender difference was found in the levels of free and total BPA in normal Korean urine samples. In contrast, there were gender differences in the levels of urinary conjugates ($p < 0.01$). Men were more likely to glucuronidate BPA than women, but women seemed to have a metabolic shunt for the sulfation of BPA. There are several reports for gender differences in the levels of BPA conjugates in animal studies. In Fisher 344 rats, female rats show higher levels of BPA–glucuronide and –sulfate than male rats [10]. A continuous exposure to BPA has an effect on lowering the expression levels of UGT 2B1, a UDP-glucuronosyltransferase, in male Wistar rats, but not in females [19].

The percent ratio of urinary conjugates of BPA with β-D-glucuronide and sulfate appears to represent a dose–response to environmental exposure to BPA (Fig. 6). Recently, Mori et al. [20] reported that there are correlations between concentrations of polychlorinated biphenyls (PCBs) and other persistent chemicals, such as dichlorodiphenylchloroethane (DDE), hexachlorobenzene (HCB), and hexachlorocyclohexane (BHC) in human umbilical cords. That is, individuals who are exposed to an environment chemical at higher levels are more likely to be exposed to other persistent chemicals at higher levels. Normal routes of the environmental exposure to endocrine disruptors may be related to foodstuffs and living areas contaminated by various environmental chemicals, including aldrin, BHC, BPA, chlordane, DDE, dichlorodiphenyltrichloroethane, dioxins (polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans), HCB, heavy metals (cadmium and lead), PCBs, and phytoestrogens [20]. Therefore, the metabolism curves of BPA might be indicative of a dose–response to gross exposure to a range of environmental chemicals and toxicants which play a role in induction and/or stimulation of the catalytic activity for the conjugation with electrophilic substances, including β-D-glucuronide and sulfate.

In conclusion, purified β-glucuronidase from *E. coli* and sulfatase from *H. pomatia* are useful for the quantification of various BPA forms. In this study, we found gender differences in the levels of urinary

BPA–glucuronide and –sulfate. The total BPA concentration in urine is a promising index for biological monitoring human exposure to BPA. Due to the limited size of the samples and the lack of data on the toxicokinetics of BPA metabolism and excretion, the important issues, particularly gender differences in the metabolic activities, were speculated. This will be the subject of further investigation with a larger size of samples from urine, blood sera, plasma, and umbilical cord blood in various cases and controls.

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