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Determination of Free and Conjugated Forms of Bisphenol A in Human Urine and Serum by Liquid Chromatography−Tandem Mass Spectrometry

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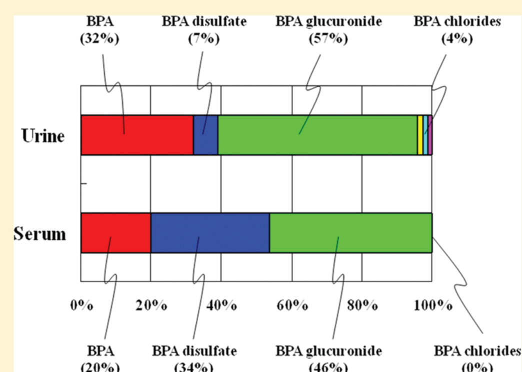
Ⓢ *Supporting Information*

ABSTRACT: Exposure of humans to bisphenol A (BPA), a widely used industrial chemical, is well-known. In humans and animals, conjugation of BPA molecule with glucuronide or sulfate is considered as a mechanism for detoxification. Nevertheless, very few studies have directly measured free, conjugated (e.g., glucuronidated), and substituted (e.g., chlorinated) forms of BPA in human specimens. In this study, free, conjugated (BPA glucuronide or BPAG and BPA disulfate or BPADS), and substituted (chlorinated BPA; mono- [BPAMC], di-[BPADC], and trichloride [BPATrC]) forms of BPA were determined in human urine and serum samples, using solid-phase extraction (SPE) and liquid chromatography−tandem mass spectrometry (LC−MS/MS) techniques. The instrumental calibration for each of the target compounds ranged from 0.01 to 100 ng/mL and showed excellent linearity ($r > 0.99$). The limits of quantification (LOQs) were 0.01 ng/mL for free BPA and 0.05 ng/mL for the conjugated and substituted BPA. Respective recoveries of the six target compounds spiked into water blanks and sample matrices (urine and serum), and passed through the entire analytical procedure, were 96 ± 14% and 105 ± 18% (mean ± SD) for urine samples and 87 ± 8% and 80 ± 13% for serum samples. The optimal recoveries of BPAG and BPADS in the analytical procedure indicted that no deconjugation occurred during the SPE procedure. The method was applied to measure six target chemicals in urine and serum samples collected from volunteers in Albany, New York. BPA and its derivatives were found in urine samples at concentrations ranging from < LOQ to a few tens of ng/mL. In serum, free and conjugated BPA were detected at sub ng/mL concentrations, whereas BPA chlorides were not detected. The urine and serum samples were also analyzed by enzymatic deconjugation and liquid−liquid extraction (LLE) for the determination of total BPA, and the results were compared with those measured by the SPE method. To our knowledge, this is the first report on the occurrence of BPAG and BPADS in human serum.



■ INTRODUCTION

Bisphenol A (BPA) is a synthetic industrial chemical that has been widely used in the production of polycarbonate plastics and epoxy resins.[1−4] BPA is commonly found in such consumer products as baby bottles, beverage cans, food containers, sports equipment, medical and dental devices, eyeglass lenses, and household electronics.[5] Studies have shown that trace amounts of BPA can leach from these consumer products.[6,7] BPA has been found not only in environmental samples, including air, water, sewage sludge, soil, dust, and foodstuffs,[2−5,9−12] but also in specimens of human bodily fluids, such as urine, blood, and other fluids (amniotic fluid, follicle fluid, saliva, and breast milk).[2,3,13−17]

Humans are frequently exposed to BPA through multiple sources,[18] and diet is considered to be the major source of exposure.[5,13] BPA is an estrogen receptor agonist.[19,20] Studies have linked BPA exposure to a variety of adverse health outcomes in humans.[1−5] BPA is metabolized in the liver to its glucuronidated form (i.e., BPA glucuronide or BPAG) and eliminated mainly through urine.[13,21] Because BPAG does not bind to the estrogen receptor, glucuronidation is considered as a mechanism of detoxification in humans.[22] However, BPAG can be deconjugated by β-glucuronidase, which is present at high concentrations in placenta, liver, kidney, and intestine.[22] The conversion of BPAG to BPA increases the potential for reactivation of BPA-induced effects. Additionally, sulfated-, hydroxylated-, and carboxylated-BPA are presumed to be minor metabolites of BPA[23−26] and have been reported to have little estrogenic activity.[27,28]

Liquid chromatography−tandem mass spectrometry (LC−MS/MS) and gas chromatography−mass spectrometry (GC-MS) have been used for the measurement of BPA and its metabolites (e.g., BPAG, hydroxylated-BPA) in biological

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specimens.[25,29−31] For sample pretreatment, liquid−liquid extraction (LLE) and solid-phase extraction (SPE) have been used in the isolation and enrichment of BPA from solid or liquid matrices.[30] Because the analytical standards of BPAG, BPA mono-(BPAS), and disulfate (BPADS) are not commercially available, BPA analysis has, thus far, been focused on the measurement of "total BPA".[24,31] The conjugated forms of BPA (including BPAG and BPA sulfates) were estimated by subtraction of free BPA (analyzed without enzymatic deconjugation of samples) from total BPA (analyzed after deconjugation of samples by β-glucuronidase) concentrations.[31,32] The commercially available β-glucuronidase, from *Helix pomatia*, usually contains a small amount of aryl-sulfatase, which can deconjugate sulfated forms of BPA.[32] Whereas BPAG is the major conjugated metabolite of BPA found in urine, other conjugated metabolites including BPA sulfates and glucosulfate-derivatives[26] have also been reported to exist in minor amounts. The earlier methods of determination of conjugated forms of BPA were not direct due to the lack of authentic analytical standards. An analytical method for the quantification of free and conjugated forms of BPA and BPA derivatives is needed for the assessment of human exposure and risk evaluation.

BPA can be chlorinated by reacting with sodium hypochlorite, which is used as a bleaching agent in the production of paper and also as a disinfectant in sewage treatments plants.[33] Chlorinated BPA analogues have been reported to occur in environmental matrices and human adipose tissues.[33−35] A recent study indicated the estrogen mimicking effects of chlorinated BPA.[36] In this study, we developed a method for the determination of free BPA, BPAG, BPADS, and three BPA chlorides, namely BPA mono-(BPAMC), di-(BPADC), trichloride (BPATrC), in human urine and serum samples, using solid-phase extraction (SPE) and LC−MS/MS detection. The samples were also analyzed for BPA, using a liquid−liquid extraction (LLE) method.[37] Differences in the concentrations of free and total BPA measured (for the SPE method, total BPA is the sum of free, conjugated, and chlorinated BPA) by the two methods are discussed.

■ MATERIALS AND METHODS

Chemicals. BPA (purity: 97%), creatinine (99%), formic acid (98.2%), acetic acid (99.9%), and β-glucuronidase from *Helix pomatia* (145 700 units/mL β-glucuronidase; 887 units/mL sulfatase) were purchased from Sigma-Aldrich (St. Louis, MO). $^{13}C_{12}$−BPA (99%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Creatinine-d_3 (99%) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). BPADS, BPAG, and three BPA chlorides (all ≥98%) were gifts from the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC (Figure 1). Ammonium acetate (98%), hydrochloric acid (HCl, 37%), ammonium hydroxide (NH_4OH, 29.5% assayed as NH_3), and all organic solvents (analytical grade) used in the experiments were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Milli-Q water was purified by an ultrapure water system (Barnstead International, Dubuque, IA). The stock solutions of BPA standards were prepared at 1 mg/mL in methanol. The stock solutions of creatinine and creatinine-d_3 were prepared at 1 mg/mL in milli-Q water. All stock solutions were stored at −20 °C. The calibration standards, ranging in concentrations from 0.01 to 100 ng/mL, were prepared from the stock solutions through serial dilution with methanol.

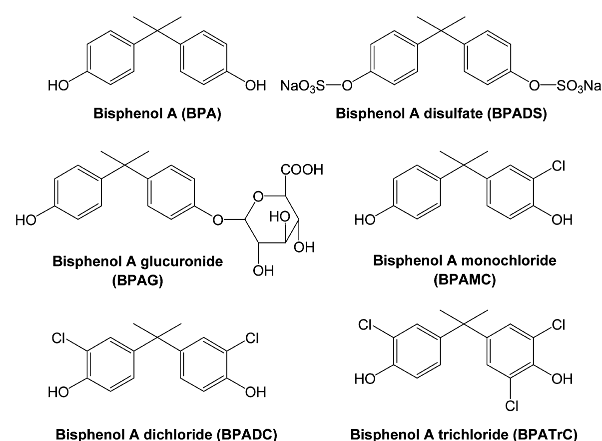

Figure 1. Chemical structures of bisphenol A (BPA) and two conjugates (BPAG and BPADS) and three derivatives (BPAMC, BPADC, BPATrC) analyzed in the present study.

Sample Collection. Thirty-one healthy volunteers provided urine samples from August to September 2011. Most of the donors were employees or students at Wadsworth Center, New York State Department of Health (NYSDOH), Albany, NY. Samples were collected from both males and females of ages ranging from 11 to 66 years, representing multiple ethnicities. Spot urine samples were collected in 50-mL polypropylene tubes and subsequently kept at −20 °C until analysis. Blood samples were obtained through venipuncture by a registered nurse at the NYSDOH. After coagulation in the sample tube (BD Vacutainer Plastic SSTTM Tube, Becton, Dickinson and Company, Franklin Lakes, NJ), blood samples were centrifuged at 1200*g* and resulting sera were transferred into new polypropylene tubes. A total of 14 serum samples were collected and kept at −20 °C until analysis. The age of donors ranged from 27 to 63 years. The detailed information about age, gender, and race are listed in Tables S1 and S2 (Supporting Information). The NYSDOH Institutional Review Board approved the study protocol for the analysis of human specimens.

Sample Preparation. Urine and serum samples were extracted following methods described elsewhere, with some modifications.[14,24,38] Briefly, after thawing at room temperature, urine or serum (0.5 mL) was transferred into a 15-mL glass tube, and 50 μL of 100 ng/mL (5 ng) internal standard ($^{13}C_{12}$−BPA), 1 mL of 1 M ammonium acetate buffer (which consisted of 7.71 g of ammonium acetate, 94 mL of milli-Q water, and 6 mL of acetic acid; pH 5.0), 0.24 mL of 1 M formic acid (3.93 mL of formic acid dissolved in 96.1 mL of milli-Q water; pH 1.0), and 1.21 mL of milli-Q water were added, for a total volume of 3 mL. For urine samples, two different SPE extraction and purification procedures were applied. An Oasis HLB cartridge (60 mg/3 cc; Waters, Milford, MA) was used for extraction of free BPA and BPA chlorides (BPAMC, BPADC, and BPATrC). The urine sample was loaded onto an HLB cartridge and preconditioned with 5 mL of methanol and 5 mL of water. The cartridge was washed with 2 mL of 0.1 N HCl and 5 mL of 10% methanol in water and eluted with 5 mL of methanol. The eluate was concentrated to 0.5 mL under a gentle stream of nitrogen and subjected to LC−MS/MS analysis.

Table 1. Elemental Composition and Ions Monitored for Bisphenol A (BPA) and Its Conjugates (BPAG and BPADS) and Derivatives (BPAMC, BPADC, and BPATrC)

target compounds	CAS no.	elemental composition	molecular weight	retention time (min)	MS/MS ions (m/z)
BPA	80-05-7	$C_{15}H_{16}O_2$	228.29	9.06	227 > 212
BPADS	10040-44-5	$C_{15}H_{14}Na_2O_8S_2$	432.38	7.05	387 > 307
BPAG	267244-08-6	$C_{21}H_{24}O_8$	404.41	7.60	403 > 113
BPAMC	74192-35-1	$C_{15}H_{15}ClO_2$	262.73	9.67	261 > 246
BPADC	79-98-1	$C_{15}H_{14}Cl_2O_2$	297.18	10.29	296 > 245
BPATrC	40346-55-2	$C_{15}H_{13}Cl_3O_2$	331.62	10.83	330 > 279

For the analysis of BPAG and BPADS, a Strata NH_2 cartridge (200 mg/3 cc; Phenomenex, Torrance, CA) mounted on a Sep-Pak C18 cartridge (200 mg/3 cc; Waters, Milford, MA) was used. The two SPE cartridges were individually preconditioned with 5 mL of methanol and 5 mL of water before being connected in series. After loading the sample, the cartridges were washed with 2 mL of 0.1 N HCl and 5 mL of 25% methanol in water and separated. The Strata NH_2 cartridge was washed with an additional 5 mL of methanol and eluted with 3 mL of 5% ammonium hydroxide (NH_4OH) in methanol. The pH of the eluate was adjusted to 7.0 with formic acid before concentration to 1 mL; this fraction (fraction 1) contained BPADS. The Sep-Pak C18 cartridge was eluted with 5 mL of methanol, and the eluate was concentrated to 1 mL; this fraction (fraction 2) contained BPAG and the internal standard, $^{13}C_{12}$−BPA.

Serum samples were extracted and purified with a Strata NH_2 cartridge mounted on top of an Oasis MCX cartridge (60 mg/3 cc; Waters, Milford, MA) as described above. Fraction 1 contained BPADS, and fraction 2 contained free BPA, BPAG, three BPA chlorides, and $^{13}C_{12}$−BPA.

Instrumental Analysis. Identification and quantification of six target compounds were performed with an Applied Biosystems API 5500 electrospray triple quadrupole mass spectrometer (ESI−MS/MS; Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA). Ten microliters of the extract was injected onto a Betasil C18 chromatographic column (100 × 2.1 mm, 5 μm; Thermo Electron Corporation, Waltham, MA), which was serially connected to a Javelin guard column (Betasil C18, 20 × 2.1 mm, 5 μm). The mobile phase, comprising methanol and 10 mM ammonium acetate at a flow rate of 300 μL/min, with a gradient starting from 15% methanol at 0 min, held for 2 min; increased to 75% methanol at 2 to 2.5 min, held for 2.5 min; then increased to 99% methanol at 5 to 10 min, held for 3.5 min; and finally reversed to 15% methanol, held for 6.5 min before the next injection. The MS/MS was operated in the electrospray negative ion multiple reaction monitoring (MRM) mode. The transitions of ions monitored are listed in Table 1. Nitrogen was used as both curtain and collision gas. The MS/MS parameters were optimized by infusing the individual compound into the mass spectrometer through a flow injection system and are summarized in Table S3 (Supporting Information). Quantification of BPA was based on the recovery of the internal standard $^{13}C_{12}$−BPA (isotope-dilution method); quantification of the other five target compounds also was based on the recovery of $^{13}C_{12}$−BPA because corresponding isotopically labeled standards were not available. The sum of the six target compounds was referred to as "total BPA." Details of quality assurance and quality control (QA/QC) are discussed below.

Analysis of Free and Total BPA by Liquid−Liquid Extraction (LLE). Free and total BPA were also measured for the same set of urine and serum specimens by the LLE method described elsewhere.[37] Briefly, after thawing at room temperature, urine or serum (0.5 mL) was transferred into a 15-mL polypropylene tube, and 5 ng of $^{13}C_{12}$−BPA was spiked as an internal standard. The sample was extracted three times, each with 3 mL of ethyl acetate. The extracts were combined, washed with milli-Q water, concentrated to near-dryness under N_2, and reconstituted with 0.5 mL of methanol before injection into LC−MS/MS. This fraction contained freely available BPA (LLE fraction 1). Another aliquot of urine or serum (0.5 mL) was digested (after the addition of 5 ng of $^{13}C_{12}$−BPA) with 1 mL of 1 M ammonium acetate containing 291.4 units of β-glucuronidase (0.77 g of ammonium acetate dissolved in 10 mL of milli-Q water with 20 μL of initial β-glucuronidase solution) at 37 °C for 12 h, and the digested sample was extracted with ethyl acetate 3 times. The final extract was concentrated, and the solvent was reconstituted with 0.5 mL of methanol. This fraction contained free plus conjugated BPA (i.e., total BPA; LLE fraction 2). BPA concentrations in samples were quantified using LC−MS/MS, as described above. The LOQ of BPA in urine and serum by the LLE method was the same as that of the SPE method. Ten nanograms of BPA was spiked into the procedural blanks (n = 5; containing milli-Q water in place of urine/serum) and in randomly selected sample matrices (n = 5); respective recoveries of BPA from spiked blanks and matrix spiked samples were 99 ± 2% and 98 ± 2% (mean ± SD) for urine samples and 104 ± 3% and 103 ± 3% for serum samples.

Analysis of Creatinine in Urine. An aliquot of urine (10 μL) was diluted with milli-Q water (∼160-fold), and 800 ng of creatinine-d_3 was added. Creatinine was analyzed with LC−MS/MS in electrospray positive ionization mode, and the MRM transitions monitored were 114 > 44 for creatinine and 117 > 47 for creatinine-d_3.[39]

Data Analysis. Data were acquired with the Analyst 1.5.1 software package (Applied Biosystems). Statistical analyses were conducted with Origin 7.5 (OriginLab Corporation, Northampton, MA). Concentrations below LOQ were substituted with a value equal to LOQ divided by the square root of 2 for the calculation of the geometric mean (GM). Differences between groups were tested by a one-way ANOVA with the Tukey test, and $p < 0.05$ was considered to be significantly different.

RESULTS AND DISCUSSION

Extraction and Purification of Samples. A solid-phase extraction method was applied for the extraction and enrichment of six BPA compounds in urine and serum samples. First, Sep-Pak C18 (200 mg/3 cc), Oasis HLB (60 mg/3 cc), and Oasis MCX (60 mg/3 cc) were tested for the extraction efficiency. Ethyl acetate, methanol, and acetonitrile were used as

elution solvents solely or in combination. Ten ng of each target compound was spiked into the randomly selected urine and serum samples ($n \geq 6$ each). Regardless of the sample type, BPA and three BPA chlorides (BPAMC, BPADC, and BPATrC) were efficiently extracted by each solvent, solely or in combination, and the extraction efficiency was similar, with recoveries of >85% for the four analytes spiked into the sample matrices. However, the recoveries of BPAG and BPADS were very low, at <20%, and did not increase with increasing solvent volume or with changing the composition of solvent mixtures. BPADS was not extracted from serum samples by the Oasis HLB cartridge. Consistent, but low, recoveries of BPADS and BPAG suggested that these compounds were either not completely extracted from the sample matrix or were transformed in the SPE procedure. We then followed a tandem cartridge (two cartridges connected in series) extraction technique, similar to that reported earlier.[24] When the tandem cartridge technique was applied, all six target analytes were efficiently recovered from serum samples: BPADS was extracted by the Strata NH_2 cartridge, mounted on top of the Oasis MCX cartridge, and the other five target compounds and internal standard ($^{13}C_{12}$−BPA) were extracted by the Oasis MCX cartridge. For the urine sample, however, BPAG, BPADS, free BPA, and three BPA chlorides were not coextracted, regardless of solvent volume and composition. Urine is a highly complex matrix, and the existence of several ionic compounds could impair coextraction of all six target compounds.[40] Therefore, we used two separate SPE procedures for the analysis of six target compounds in urine samples: (1) a tandem cartridge (Strata NH_2 cartridge connected with a Sep-Pak C18 cartridge) was utilized for the analysis of BPAG and BPADS, in which BPADS was detected in the elute of a Strata NH_2 cartridge and BPAG was in the elute of a Sep-Pak C18 cartridge; and (2) an Oasis HLB cartridge was utilized in the analysis of free BPA, BPAMC, BPADC, and BPATrC.

Deconjugation of BPAG and BPADS. Conjugated BPA forms, such as BPAG, can be enzymatically deconjugated in some tissues that contain β-glucuronidase.[22] The chemical bond linking BPA with glucuronic acid or sulfate is relatively weak, as compared with carbon−carbon or carbon−oxygen bonds in a BPA molecule. The potential for deconjugation of BPAG and BPADS during extraction, enrichment, and purification steps was examined by spiking three levels (10, 50, and 100 ng) of these compounds individually in urine and serum samples (n = 3 each) and passed through the entire analytical procedure. Both BPAG and BPADS were detected in the final extracts. The matrix spike recoveries of BPAG in urine and serum were 94 ± 12% and 106 ± 12%, respectively; the corresponding values of BPADS were 103 ± 16% and 113 ± 17%, respectively. No free BPA was found in these samples. These results demonstrated that no deconjugation of BPAG and BPADS occurred during sample extraction and purification steps.

Detection Limits, Procedural Recoveries, and Procedural Blanks. Instrumental calibration was verified by injecting 10 μL of 0.01 to 100 ng/mL standards of the six target compounds, which showed excellent linearity (regression coefficient = r > 0.99). The limit of detection (LOD) and LOQ were calculated as 3 times ($3S$) and 10 times ($10S$) the standard deviations (S) of five replicate analyses, using the lowest calibration standard (0.01 ng/mL). The calculated LOD and LOQ, respectively, were 0.003 and 0.01 ng/mL for free BPA and 0.02 and 0.05 ng/mL for conjugated and substituted forms of BPA.

Prior to the analysis of samples, a recovery test was conducted, through the spiking of all three concentrations (10, 50, and 100 ng) of each of the six target compounds into the sample matrices, with subsequent passage through the entire analytical procedure ($n \geq 6$). For the urine sample, the recoveries of target compounds ranged from 78 to 123%, with a relative standard deviation (RSD) of 5−16%, 76 to 115% (RSD 3−11%), and 78 to 129% (RSD 2−19%) for the low (10 ng), medium (50 ng), and high (100 ng) concentrations, respectively. The corresponding values for serum samples were 72 to 118% (RSD 5−11%), 83 to 138% (RSD 3−15%), and 76 to 123% (RSD 8−18%) for low, medium, and high concentrations, respectively. These results indicated that no significant loss of target compounds occurred during the SPE procedure.

For every batch of 20 samples analyzed, a procedural blank, a spiked blank, and a pair of matrix spiked samples (10 ng) were processed. The procedural blank, containing milli-Q water in place of urine/serum, was analyzed as a check for interferences or laboratory contamination. Trace levels of free BPA (approximately 0.006 ng/mL) were found in procedural blanks in some batches, and background subtraction was performed in the quantification of concentrations in samples. No other target compounds were found in procedural blanks. The respective recoveries of six target compounds spiked into water blanks and sample matrices were 96 ± 14% and 105 ± 18% (mean ± SD) for urine samples and 87 ± 8% and 80 ± 13% for serum samples.

Analysis of Urine and Serum Samples. Thirty-one urine and fourteen serum samples, collected from Albany, NY, were analyzed by the SPE method developed in this study. Typical MRM chromatograms of six target compounds detected in human urine/serum are shown in Figure 2. Free BPA and BPAG were frequently found in urine samples, at concentrations ranging from < LOQ to 18.7 ng/mL (detection rate: 97%; GM: 0.701 ng/mL, 0.713 μg/g creatinine) and from < LOQ to 65.2 ng/mL (87%; 2.16 ng/mL, 2.20 μg/g creatinine), respectively; BPADS concentrations ranged from < LOQ to 6.16 ng/mL (36%; 0.108 ng/mL, 0.110 μg/g creatinine). Trace levels of BPA chlorides (BPAMC, BPADC, and BPATrC) were found in <20% of the urine samples, with GM concentration of <0.06 ng/mL (0.06 μg/g creatinine) (Table 2). BPA chlorides were not detected in serum samples (Table 2). The other three target compounds were detected in approximately half of the serum samples (detection rate: ∼ 50%) at concentrations of < LOQ to 0.588 ng/mL (GM: 0.035 ng/mL) for free BPA, < LOQ to 1.77 ng/mL (0.124 ng/mL) for BPADS, and < LOQ to 11.9 (0.124 ng/mL) for BPAG (Table 2). No significant differences in total BPA concentrations between men and women were found. The total BPA concentration in urine from Caucasians was significantly higher than that found in urine from Asians (Figure S1 in Supporting Information).

Very few studies have directly measured the concentrations of BPA conjugates (i.e., BPAG and BPADS) or BPA chlorides in human urine and serum samples. To our knowledge, no earlier studies have reported the occurrence of BPAG in human serum and BPADS in urine or serum. A study from Korea[41] determined BPA and its metabolites, BPAG and BPA sulfate (BPAS), in urine samples by an indirect method involving treatment of samples with or without β-glucuronidase and sulfatase. The Korean study showed the presence of gender

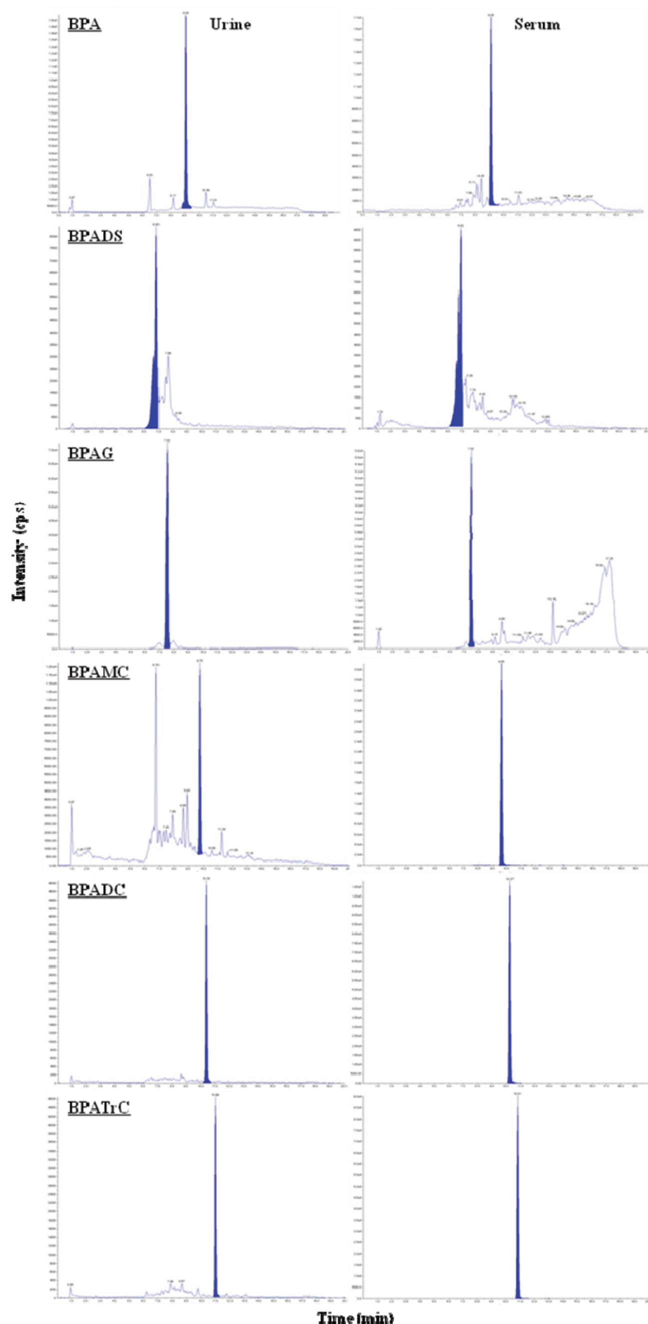

Figure 2. LC−MS/MS chromatograms for bisphenol A (BPA) and its conjugates and derivatives found in human urine and serum. The concentrations of BPA, BPADS, BPAG, BPAMC, BPADC, and BPATrC in the final extracts were 1.66, 4.12, 4.23, 0.568, 0.697, and 0.433 ng/mL for urine samples, and 0.567, 3.23, 9.21, 8.9, 11.7, and 10.5 ng/mL for serum samples, respectively. Because BPA chlorides were not detectable in all serum samples, three chromatograms (three lower right panels) for BPA chlorides were adopted from spiked matrices (10 ng).

differences in the levels of urinary BPA conjugates. BPAG concentrations (2.34 ± 0.85 ng/mL, mean ± SE) in urine samples of Korean men ($n = 15$) were significantly higher than those (1.00 ± 0.34 ng/mL) of Korean women ($n = 15$), whereas BPAS concentrations (0.49 ± 0.27 ng/mL) in men were lower than those (1.20 ± 0.32 ng/mL) in women. By using a similar method of sample preparation, Ye et al.[42] reported BPAG and BPAS concentrations in urine samples collected from 30 U.S. volunteers, at <0.3−19.0 ng/mL (mean: 3.1 ng/mL), and <0.3−1.8 ng/mL (mean: 0.5 ng/mL), respectively.

Our results suggest that BPAG is the dominant species, representing 57 ± 34% (mean ± SD) of the total BPA concentration in urine, followed, in decreasing order, by "free" BPA (32 ± 31%), BPADS (7 ± 14%), BPAMC (1.8 ± 6.8%), BPADC (1.3 ± 4.6%), and BPATrC (1.2 ± 4.4%) (Figure 3). No gender or ethnicity-related differences in the profiles of BPA in urine were found (Figure 3). BPAG is also the dominant species in serum samples, accounting for 43 ± 41% of the total BPA concentration, followed by BPADS (38 ± 38%) and free BPA (19 ± 30%). Our results also showed that there was no significant gender difference in the concentrations of BPAG in either urine (GM, 2.22 μg/g creatinine for men versus 2.17 μg/g creatinine for women; $p > 0.05$) or serum (GM, 0.171 ng/mL for men versus 0.256 ng/mL for women; $p > 0.05$). The sum concentration of six target compounds in urine (GM: 5.40 ng/mL) was approximately 1 order of magnitude higher than that (GM: 0.537 ng/mL) in serum (Table 2).

SPE versus LLE. The urine and serum samples that were analyzed using the SPE method, as described above, were analyzed for free (LLE fraction 1) and total BPA (LLE fraction 2) by LLE with ethyl acetate.[37] Free BPA was detected in 30 of 31 (97%) urine samples at concentrations ranging from < LOQ to 2.24 ng/mL (GM: 0.364 ng/mL), and total BPA was detected in all urine samples at concentrations ranging from 0.364 to 8.29 ng/mL (GM: 1.07 ng/mL; Figure S2 in Supporting Information). The creatinine-adjusted urinary concentrations of free and total BPA, determined by LLE, were in the range of < LOQ to 11.8 μg/g (GM: 0.370 μg/g) and 0.223−11.8 μg/g (1.09 μg/g), respectively (Figure S2). Free and total BPA were found in all serum samples ($n = 14$) at concentrations ranging from 0.020 to 0.100 ng/mL (GM: 0.049 ng/mL) and from 0.036 to 0.121 ng/mL (GM: 0.075 ng/mL), respectively (Figure S2).

The concentration of total BPA in urine (GM: 5.49 μg/g creatinine), determined by the SPE method, was higher than that (GM: 1.09 μg/g creatinine) determined by the LLE method ($p < 0.001$, one-way ANOVA), whereas the concentrations of free BPA were comparable between the two methods (GM: 0.713 versus 0.370 μg/g creatinine; $p > 0.05$, Figure S2). However, no significant differences were found in the free and total BPA concentrations in serum, although the GM concentration of total BPA (0.537 ng/mL) determined by the SPE method was slightly higher than that (0.075 ng/mL) determined by the LLE method (Figure S2). Although many studies have reported the occurrence of BPA in human urine and serum, there have been considerable discrepancies with regard to the extraction efficiency of the analytical methods.[22,31] Our results suggest that no obvious differences were found in the levels of free and total BPA in serum samples between the SPE and the LLE methods. For urine samples, however, the SPE appears to be more suitable than the LLE, especially for the speciation analysis,[31] because several factors such as solvent extraction efficiency and matrix interferences encountered in the LLE method can reduce/suppress actual concentrations found in the samples.

Conjugation with β-glucuronide and sulfate can reduce the biological activities of BPA and subsequently facilitate the excretion via urine.[22,42] Therefore, analysis of free, conjugated, and derivative forms of BPA in biological specimens is

Table 2. Concentrations of Free Bisphenol A (BPA) and Its Conjugates and Derivatives in Urine and Serum Samples Determined by SPE Method and Compared with Liquid−liquid Extraction (LLE) Method

	urine					serum		
	unadjusted concentration (ng/mL)		creatinine-adjusted concentration (μg/g)					
compounds	range	GM	range	GM	detection rate	range	GM	detection rate
free BPA[a]	<LOQ−18.7	0.701	<LOQ−12.8	0.713	96.8	<LOQ−0.588	0.035	57.1
BPADC	<LOQ−1.06	0.048	<LOQ−0.617	0.049	19.4			ND
BPADS	<LOQ−6.16	0.108	<LOQ−3.99	0.110	35.5	<LOQ−1.77	0.124	50.0
BPAG	<LOQ−65.2	2.16	<LOQ−38.3	2.20	87.1	<LOQ−11.9	0.204	50.0
BPAMC	<LOQ−1.68	0.055	<LOQ−2.31	0.055	16.1			ND
BPATrC	<LOQ−0.675	0.047	<LOQ−0.394	0.047	19.4			ND
total (SPE)[a]	0.222−66.2	5.40	0.093−40.4	5.49		<LOQ−13.8	0.537	
free BPA (LLE)[b]	<LOQ−2.24	0.364	<LOQ−11.8	0.370	96.8	0.020−0.100	0.049	100.0
total (LLE)[b]	0.364−8.29	1.07	0.223−11.8	1.09	100.0	0.036−0.121	0.075	100.0

[a]Free and total BPA data were from SPE extraction. [b]Free and total BPA data were from liquid−liquid extraction.

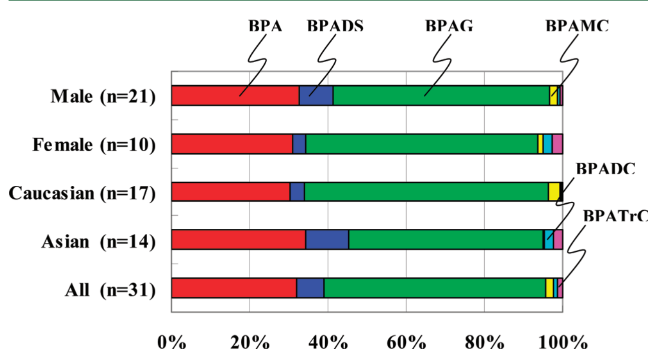

Figure 3. Composition profile of different forms of bisphenol A (BPA) in human urine.

important for exposure and risk assessments.[41] To our knowledge, this is the first study describing the occurrence of BPADS and BPA chlorides in human urine and serum samples. The newly developed analytical procedure is suitable for the analysis of different species of BPA. Further studies should focus on determination of different forms of BPA in human bodily fluids, including amniotic fluid, follicle fluid, and breast milk.

■ ASSOCIATED CONTENT

Supporting Information

Two tables showing detailed information on age, gender, and race of donors of urine and serum samples; one table showing MS/MS parameters optimized for analysis of BPA and its conjugates and derivatives in urine and serum samples; two figures showing comparison of the concentrations of free and total BPA for urine and serum samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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