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Comparison of Elisa- and LC-MS-Based Methodologies for the Exposure Assessment of Bisphenol A

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Several types of methods, mainly liquid chromatography (LC), have been used for the analysis and assessment of bisphenol A (BPA) in human biological samples. Enzyme-linked immunosorbent assay (ELISA) is also being used for BPA measurement. In this study, we verified whether ELISA is suitable for measuring human samples, namely, serum and urine, by comparing the ELISA results with those obtained by liquid chromatography with multichannel colometric electrochemical detection (LC/ECD) and liquid chromatography coupled to a mass spectrometer (LC/MS/MS). Results of the measurement with LC/ECD showed urinary BPA concentrations to be 1.92 [1.45] ± 1.99 (mean [median] ± standard deviation) ng BPA/mL without the correction of urine volume and 1.20 [0.90] ± 1.10 μg BPA/g creatinine; however, in serum, free and total BPA were not detected. In both samples, a good correlation of the values with the methods was not found. ELISA is one of the powerful measurement methods, since it is convenient and useful for screening bulk quantities. At this point, however, ELISA is not suitable for BPA measurement in human samples because of low levels of BPA in human samples, matrix effect, and specificity of anti-BPA antibody.

Keywords Bisphenol A, ELISA, LC/MS, LC/ECD

INTRODUCTION

Bisphenol A [2,2-(4,4-dihydroxydiphenol)propane] (BPA) is an estrogenic compound that is widely used in the manufacture of polycarbonate plastics, which serve as containers for foods and beverages and as a constituent of dental sealants. The core structure of BPA resembles that of the natural estrogen estradiol, and BPA is known to have weak estrogen-like activity. A low dose of BPA induces in vitro proliferation of estrogen-sensitive tissues (Takeuchi and Tsutsumi 2002; Olea et al. 1996). In rodents, developmental exposure to BPA caused a decrease in epididymal weight (vom Saal et al. 1998) and daily sperm production (Sakaue 2001), reduction in pituitary luteinizing hormone secretion, and decrease in steroidogenic enzyme gene expression in Leydig cells (Akingbemi et al. 2004). Moreover, BPA exposure causes meiotic aneuploidy (Hunt et al. 2003) and affects the differentiation of oligodendrocyte precursor cells (Seiwa et al. 2004).

Several types of methods, mainly liquid chromatography, have been used for the analysis and assessment of BPA in human biological samples (Inoue et al. 2003). Enzyme-linked immunosorbent assay (ELISA) is also being used for the measurement of BPA in urine (Usuki et al. 2000), serum (Yamada et al. 2002; Takeuchi and Tsutsumi 2002), semen (Inoue et al. 2002), and ovarian follicular fluids (Ikezuki et al. 2002). It is believed that human samples are affected more easily by the matrix effect than environmental samples. In this study, we verified whether ELISA is suitable for measuring human samples, namely, serum and urine, by comparing the ELISA results with those obtained by liquid chromatography with multichannel colometric electrochemical detection (LC/ECD) and liquid chromatography coupled to a mass spectrometer (LC/MS/MS).

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MATERIALS AND METHODS

Samples and Pretreatment

Blood (30 mL) and urine (50 mL) were collected from 52 volunteers (age 22–51, 21 men and 31 women) between July and September 2004. Serum was separated by centrifugation. The samples were stored at –20°C until use. This study has been approved by the "Congress of Medical Bioethics" of Chiba University.

Reagents

BPA standard, BPA-glucuronide, and β-glucuronidase (type-2) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Frontier Science Co., Ltd. (Ishikari, Japan), and Nippon Biotest Laboratories Inc. (Tokyo, Japan), respectively.

Determination of Serum and Urinary BPA (Total BPA and Free BPA) by High-Perfomance Liquid Chromatography (HPLC)

Serum and urinary BPA concentration (total BPA and free BPA) were measured by HPLC according to a previous report (Gondoh et al. 2003). Relative standard deviation was less than 5%, a recovery rate was 91.6% to 102.3%, and the detection limit was 0.2 ng/mL.

One milliliter of 0.03 mol/L acetate buffer (pH 4.5) and 25 μL of β-glucuronidase (1,050 units) were added to 0.5 mL of serum or urine, and the mixture was hydrolyzed by incubating at 37°C for 16 hours (no hydrolysis for free BPA). The BPA in the sample was extracted with Isolute Multimode Extraction Column (500 mg, International Sorbent Technolgy Ltd., USA) and then analyzed by HPLC using column switching. The HPLC system consisted of a LC-10A vp series (Shimadzu Co., Kyoto, Japan), Hypercarb precolumn (4.6 × 35 mm, Thermo Electron Co., USA), an Inertsil ODS-3V main column (4.6 × 250 mm, GL Sciences, Tokyo, Japan) set in the column oven at 60°C, and an ECD100 (Eicom Co., Kyoto, Japan) for electric chemical detection (ECD). The mobile phase used for the precolumn was a mixture of 0.016% acetic acid and water (1:3 v/v) and that for the main column was a mixture of 6.9 mmol/L phosphate buffer, ethanol, and acetonitrile (11:1:7 v/v/v). The flow rate was set at 0.8 mL/min.

Determination of Urinary BPA-Glucuronide by Liquid Chromatography Tandem Mass Spectrometry

Urinary BPA-glucuronide was measured by LC/MS/MS according to a previous report (Miyagawa et al. 2004). Relative standard deviation was less than 7%, a recovery rate was 90.8% to 110.4%, and the detection limit was 1 pmol/mL.

A volume of 0.2 mL urine was diluted threefold with 25% methanol containing 0.25% acetic acid. BPA-glucuronide in the sample was then analyzed by LC/MS/MS. The LC system consisted of an Agilent 1100 series (Agilent, USA), Pegasil ODS precolumn (2.0 × 150 mm, Senshu Scientific, Tokyo, Japan), and an Inertsil ODS-3 main column (2.0 × 150 mm, GL Sciences, Tokyo, Japan) set in the column oven at 46°C. The column switching method was employed. The mobile phase used for the precolumn was a mixture of 10 mol/L acetate buffer and acetonitrile (4:1 v/v) and that for the main column was a mixture of 0.01% acetic acid, tetrahydrofuran, and acetonitrile (2:2:1 v/v/v). The flow rate was set at 0.18 mL/min. BPA-glucuronide separated by LC was then analyzed with Quattro Ultima ESI-tandem mass spectrometry (Micromass, Manchester, UK). BPA-glucuronide was quantitatively determined by employing multiple reaction monitoring (MRM, precursor ion: m/z = 403, production ion: m/z = 113) with a collision energy of 15 eV and cone voltage of 60 V.

BPA Measurement by ELISA

An ELISA was performed using ELISA kits on the market by manufacture's hand: BPA ELISA Eiken kit (Kit Eiken) (Eiken Chemical Co.Ltd., Tokyo, Japan), EcoAssay Bisphenol A kit (Kit Otsuka) (Otsuka Pharmaceutics, Tokyo, Japan) and Supersensitive BPA ELISA for Biological Samples (Kit Env) (Japan EnviroChemicals, Ltd., Osaka, Japan). The detection limit of all ELISAs was 0.2 ng/mL. The anti-BPA antibodies included in these kits recognize not only free BPA, but also glucuronate conjugate.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2003.

RESULTS

BPA Concentration in Urine

Low levels of free BPA were detected in only 2 of the total 52 cases by LC/ECD: 0.24 and 0.35 ng/mL. Total BPA was detected in 50 of 51 cases (measurement could not be performed in one case due to interference substances) by LC/ECD and the concentration was found to be 1.92 [1.45] ± 1.99 (mean [median] ± standard deviation) ng BPA/mL without the correction of urine volume (Fig. 1A) and 1.20 [0.90] ± 1.10 μg BPA/g creatinine with the correction of urinary creatinine concentration (Fig. 1B). This implies that the urine total BPA (glucuronidated BPA plus unconjugated BPA) concentrations were essentially equal to the glucuronidated BPA concentration.

Monoglucuronidated BPA concentrations in 10 urine samples were measured directly by LC/MS/MS. Of these, total BPA (≈glucuronidated BPA) concentrations were determined by LC/ECD, and both values were compared in order to validate LC/ECD values. Both values showed very good correlation at least in the range of 2 to 43 pmol/mL (≈0.5–10 ng unconjugated BPA/mL determined by LC/ECD); the correlation coefficient between them was 0.998 and the slope of the regression line was 0.963 (Fig. 2). The results suggested that LC/ECD is useful

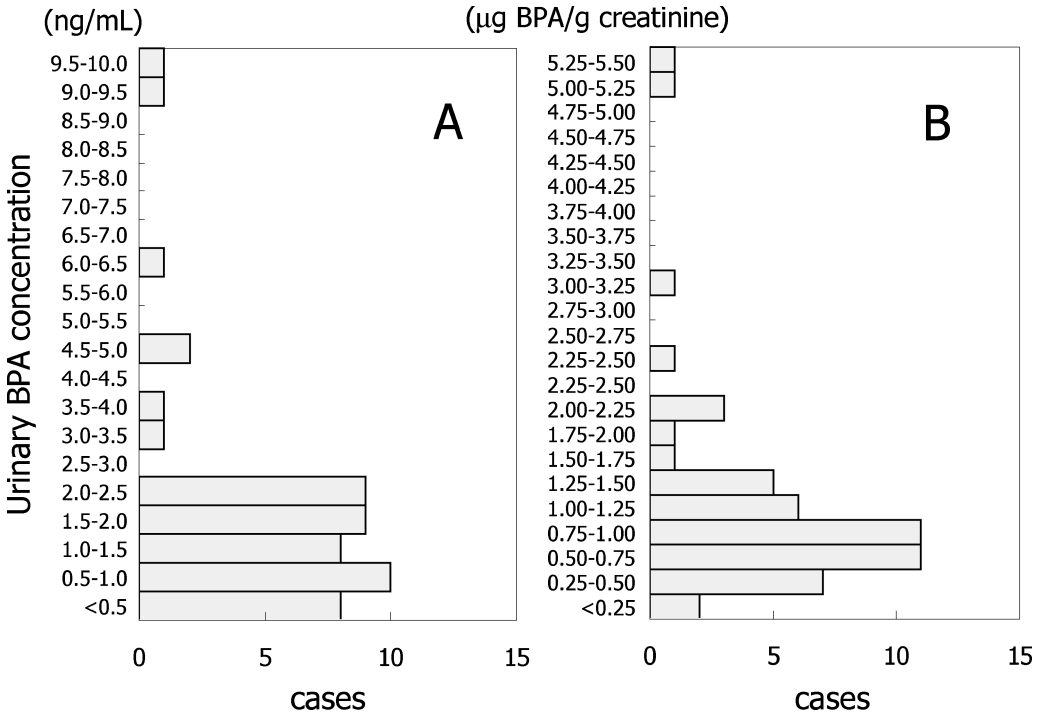

FIG. 1. Distribution of urinary BPA concentration. BPA concentration measured by LC/ECD has been shown in ng BPA/mL (A) and μg BPA/g creatinine (B).

in determining the quantity of BPA and that most of the urinary BPA is monoglucuronidated in humans.

On the other hand, ELISA was not applicable for the measurement of BPA in urine due to the matrix effect. When we measured the urinary total BPA concentrations as a reference, the values measured by Kit Eiken, Kit Env, and Kit Otsuka were observed to be 15.9 [14.1] $\pm$ 9.9, 16.7 [10.7] $\pm$ 19.5, and 18.6 [10.8] $\pm$ 23.7 ng/mL, respectively (data not shown). Not only the correlation coefficients between LC/ECD and ELISAs, but also those among the ELISAs were low (Table 1).

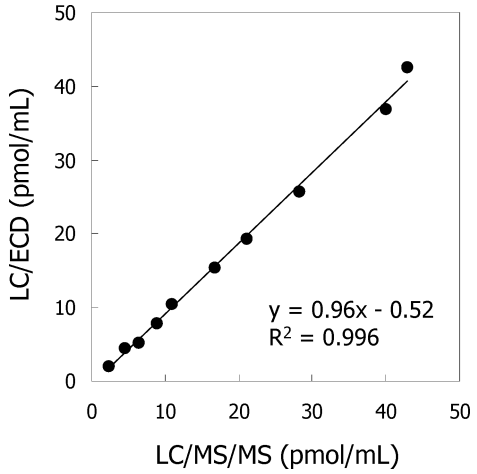

FIG. 2. Relationship between urinary BPA concentration measured by LC/MS/MS and that by LC/ECD. The value by LC/ECD is for the total BPA concentration after the samples were treated with β-glucosidase; on the other hand, the value by LC/MS/MS is for monoglucuronidated BPA.

BPA Concentration in Serum

The total BPA concentrations in almost all the serum samples were under the detection limit of Kit Eiken as well as LC/ECD. On the other hand, the other two kits detected the total BPA in almost all samples; Kit Otsuka, 0.66 [0.58] $\pm$ 0.29 ng/mL, and Kit Env, 0.71 [0.52] $\pm$ 0.49 ng/mL. In some cases, free BPA concentrations were higher than the total BPA concentrations, and the correlation coefficient between Kit Otsuka and Kit Env was 0.06. This may be attributed to the fact that glucuronidase or the ingredients of the reaction mixture for the glucuronidase affected the binding affinity of the anti-BPA antibody in the ELISA kits.

TABLE 1
Correlation coefficients of urinary BPA concentrations measured by LC/ECD and three ELISA kits

	LC/ECD	Kit Eiken	Kit Otsuka	Kit Env
LC/ECD	—	0.62	0.30	0.15
Kit Eiken	0.62	—	0.59	0.57
Kit Otsuka	0.30	0.59	—	0.55
Kit Env	0.15	0.57	0.55	—

DISCUSSION

Free and total BPA were not present in serum; on the other hand, free BPA was detected in 4% of the urine samples, and total BPA in all the urine samples was detected by LC/ECD. Urinary BPA concentrations (1.92 [1.45] ± 1.99 ng BPA/mL without the correction of urine volume and 1.20 [0.90] ± 1.10 μg BPA/g creatinine) were in the same range as that in previous reports (Calafat et al. 2005; Ouchi and Watanabe 2002; Matsumoto et al. 2003). A very good correlation between total BPA concentration measured by LC/ECD and monoglucuronidated BPA concentration measured by LC/MS/MS suggested that BPA in almost all human urine samples was conjugated as monoglucuronidated BPA. These results suggested that humans are currently exposed to low levels of BPA.

ELISA kits are also validated by recovery tests, correlation test between instrumental analysis, etc. (Inoue et al. 2002; Ohkuma et al. 2002; Usuki et al. 2000), but the range of validation is currently relatively high for human samples. ELISA is not currently suitable for measuring BPA in human samples. This is because BPA concentrations in human samples are very low (almost all the serum samples had <0.2 ng BPA/mL and the urine samples had <2.5 ng BPA/mL) and near the detection limit of ELISA. Further, ELISA is easily affected by irregular noise such as matrix effects, particularly on the urine samples and nonspecific binding of anti-BPA antibody. Cross-reactivity of the anti-BPA antibodies with substances having structures similar to BPA has been well documented (Ohkuma et al. 2002), but the antibody cross-reacts with equol, one of the phytoestrogens, and unknown substances in urine (Hirobe et al. 2004).

Although both LC/ECD and LC/MS/MS methods show high sensitivity and accuracy, the cost is expensive and the handling is time consuming. On the other hand, ELISA is one of the powerful measurement methods since it is convenient and useful in screening bulk quantities. At this point, however, ELISA for BPA measurement is not suitable for the human samples as described above. To apply ELISA to human samples, it is necessary to develop a pretreatment method optimized to the type of samples and to understand the materials that cross-reacted with the anti-BPA antibodies. In addition, researchers should select measuring methods carefully.

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