Development of Analytical Method for Determining Trace Amounts of BPA in Urine Samples and Estimation of Exposure to BPA

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Abstract

We have developed a reliable analytical method for determining trace amounts of bisphenol A (BPA), a suspected endocrine disruptor, in urine samples by use of GC/MS so that daily exposure of human bodies to BPA can be estimated. We administered BPA-d\textsubscript{16} (100 \(\mu\)g) to volunteers in order to conduct an excretion experiment and found that the BPA was absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine, in an amount of almost 100\% in 24 hours. The results suggest that determining the BPA content of urine samples enables estimation of the exposure to BPA.

The results of our analysis of urine from adults show that the average total BPA concentration was 0.82 ng/ml (0.14–5.47 ng/ml, \(n=91\)) and that the average free BPA concentration was 0.08 ng/ml (0.01–0.27 ng/ml, \(n=11\)). Also, from a determination of whole-day urine samples, the exposure to BPA was estimated to be 1.68 \(\mu\)g/day (0.48–4.5 \(\mu\)g/day, \(n=22\)) on the average.

Key words: BPA, endocrine disruption, GC/MS, exposure

INTRODUCTION

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane; BPA], a suspected endocrine disruptor, has been widely used in large amounts as a raw material for polycarbonate, epoxy resin, phenol resin, polyester, antioxidant, and a stabilizer for vinyl chloride. This has led to a great concern about human exposure to BPA and potential effects on the human body. BPA does not exhibit high acute toxicity; LD\textsubscript{50} in rats is 3250 mg/kg (oral)\textsuperscript{b}. Some reports have stated that BPA is an endocrine disruptor: BPA shows a weak estrogenic activity in an ESCREEN test on the cellular level\textsuperscript{c}, and promotes the multiplication of human MSF-7 breast cancer cells\textsuperscript{d}. In animal tests, upon exposure to BPA female mice show a decrease in the number of litters delivered and the number of offspring per litter, as well as a decrease in birth weight, and male mice show a decrease in the number of offspring, an increase in the weight of seminal vesicle, and a deterioration in the motility of sperm cells\textsuperscript{e}.

Conventionally, exposures to a substance via differ-
ent pathways are estimated from the concentrations of the substance in food, air, water, and the like, as well as the amounts of intake, and the estimated amounts are added together in order to estimate the amount of exposure to a chemical substance. However, the conventional method has a drawback in that a large amount of work is required. If the exposure to BPA can be estimated from the amount of excreted BPA in urine, the amount of work and cost will be substantially reduced.

Reported methods for determining BPA include GC/MS5-8, LC-MS9-20, HPLC13,14, and ELISA15. However, most of the instruments used for collection, storage, and clean-up of samples are composed of high polymer materials. Thus they may contaminate samples, resulting in an overestimation when trace amounts of BPA in biological samples are to be determined. Accordingly, urgent demand exists for a highly reliable and sensitive analytical method for determining BPA that is free from the risk of contamination.

In our method, the BPA in urine is subjected to an enzymolysis and then to a solid phase extraction by use of a C₈ cartridge. The extract is trimethylsilylated (TMS), and the TMS derivative obtained is purified with a florisol cartridge, and then determined using GC/MS–SIM. We aim to estimate the human daily exposure to BPA by determining the concentration and amount of excreted BPA in urine samples by employing our method.

**EXPERIMENTAL SECTION**

**Reagents**

BPA was a product of Wako Pure Chemicals. ¹³C-BPA was purchased from Cambridge Isotope. Purified water was obtained by filtering super pure water through an activated charcoal cartridge. (β-glucuronidase was a product of Wako Pure Chemicals intended for biochemical use. C₈ cartridge used was a Supelclean ENVI-18 (0.5 g) from Supelco, and the florisol cartridge was a Supelclean ENVI-florisol (0.5 g) from Supelco. BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) was purchased from GL Sciences, Inc. Methanol, ethyl acetate, n-hexane, and acetone were products of Kanto Kagaku intended for analysis of agricultural chemical residues. Phosphoric acid was a product of Wako Pure Chemicals of special grade.

**Apparatus**

The apparatus used were an ultrasonic washer (Shibata SU-3TH), a vacuum pump (Yamato WP-25), a solid phase extraction equipment (Supelco VISIPREP DL), a super pure water production equipment (Millipore Milli-Q-ST), and a GC/MS (JEOL GC-mate).

**Collection of urine samples**

All the urine samples were collected after the procedure of informed consent.

Exposure experiment: A subject orally consumed 100 ml of a drink containing 100 µg of BPA-d₁₆, and urine samples were collected at predetermined intervals for 26.5 hours after intake (n=1). Twelve male and thirteen female volunteers each orally consumed 100 ml of a drink containing 50 µg of BPA-d₁₆, and urine samples were collected for 5 hours after intake. Urine samples were collected from 46 male and 23 female volunteers at least twice, and samples from the same subject were combined.

Whole-day urine samples were collected from 11 male and 11 female volunteers.

**Method**

Total BPA: 100 ml of a urine sample was placed in a conical flask equipped with a ground stopper, and 100 µl of β-glucuronidase solution and 0.1 mg of ¹³C-BPA were added to the flask. The mixture was subjected to an enzymolysis at 37 °C for 90 minutes. To the resultant mixture, 1 ml of 7.5 M phosphoric acid was added in order to adjust pH to 3 or lower. The mixture thus obtained was loaded onto a C₈ cartridge that had been activated with 5 ml of methanol and 10 ml of purified water, to extract BPA. After the cartridge was washed with 10 ml of 10 % methanol, 3 ml of methanol was added to elute BPA. The eluate was collected in a 100 ml eggplant-shaped flask. To the eluate, 20 ml of ethyl acetate was added, and concentrated to dryness by use of a rotary evaporator. To the flask, 200 µl of BSTFA and 2 ml of acetone were added, and the mixture was allowed to stand overnight to be trimethylsilylated, and the stripped of acetone by use of a rotary evaporator. To the resultant mixture, 2 ml of n-hexane was added and dissolved by use of an ultrasonic washer. The resultant mixture was loaded onto a florisol cartridge that had been pre-washed with 5 ml of n-hexane, and the eluate was collected in a test tube. Subsequently, the flask was
washed twice with 2 ml of n-hexane each time, and the mixtures obtained were loaded onto the cartridge. The eluate was added to the previously obtained eluate in a test tube. The resultant eluate was concentrated to 1 ml by blowing nitrogen gas, and then subjected to an analysis by GC/MS-SIM.

Free BPA: To 200 ml of a urine sample, which had not been subjected to enzymolysis, 2 ml of 7.5 M phosphoric acid and 0.05 μg of 13C-BPA were added. The subsequent procedure was the same as that described above for total BPA.

Calibration curve: BPA was placed in test tubes stepwise in an amount of 10~200 ng, and 100 ng of 13C–BPA was added as a surrogate, and then 200 μl of BSTFA was added. The volume of the resultant mixture was increased to 1 ml by adding acetone. The mixture obtained was allowed to stand overnight and then subjected to a GC/MS-SIM. A calibration curve was constructed on the basis of area ratios relative to 13C–BPA.

**GC/MS conditions**

GC separation was carried out with an HP-5890 series II. GC conditions were as follows: column DB-5MS, inner diameter 0.32 mm, length 30 m, film thickness 0.25 μm; column temperatures 70°C (2 min) —20°C/min—150°C—10°C/min—300°C (5 min), inlet port temperature 250°C; carrier gas He, flow velocity 1 ml/min; injection method splitless, purge off 1 min.

MS analysis was carried out with an JEOL GCmate. The SIM conditions were as follows: ion source temperature 230°C; ionization voltage 70V; monitor ions (m/z), BPA (357, 372), BPA-d16 (368), and 13C–BPA (369).

**RESULTS AND DISCUSSION**

**Enzymolysis of conjugates**

Insoluble compounds taken into the body are converted into highly water-soluble glucuronide conjugates during the metabolic process before excretion. Phenol compounds are believed to be excreted mainly as a glucuronide conjugate. Thus, we studied the amount of β-glucuronidase and the incubation time required for decomposition of the glucuronide conjugate of BPA. The results show that the decomposition was almost completed by addition of 50 μl of β-glucuronidase to 100 ml of urine followed by incubation at 37°C for 60 minutes. In our analysis using real samples, we chose to add 100 μl of β-glucuronidase and perform enzymolysis at 37°C for 90 minutes, in order to provide a margin of safety.

**Extraction, washing, and elution using the C₁₈ cartridge**

Phenol compounds are generally subjected to solid phase extraction at a pH of 3 or lower. The C₁₈ cartridge used in our analysis allows extraction under this condition. In our analysis, after the cartridge was washed with 10% methanol, an addition of 3 ml of methanol allowed elution of BPA. No elution of BPA from the cartridge was observed.

**Trimethylsilylation of BPA**

Since BPA is absorbed in a GC column when subjected to direct analysis at low concentrations, determination of BPA at extremely low concentrations is difficult and requires derivatization. Commonly used forms of derivatization include pentafluorobenzylization (PFB) and trimethylsilylation (TMS). For our analysis, since we use urine samples which can be collected in a large amount, we chose TMS. Since urine samples are considered to contain a variety of substances that act on BSTFA, 200 μl of BSTFA was added to each sample, and the resultant mixture was allowed to stand overnight. Fig. 1 shows the mass spectrum of BPA-TMS, and Fig. 2 shows the mass spectrum of 13C–BPA-TMS.

**Clean-up using the florisil cartridge**

Since urine samples contain a variety of metabolites, trimethylsilylation of extracts was found to produce a tar-like substance and require a clean-up treatment for determination. We studied a clean-up using a florisil cartridge and found that n-hexane caused trimethylsilylated compounds of BPA to elute easily and that clean-up was achieved by merely allowing a reagent dissolved in n-hexane to pass through the cartridge.

**Excretion of BPA**

In order to study the kinetics of excretion of BPA, 100 ml of a drink containing 100 μg of BPA-d₁₆ was administered, and urine samples were collected at predetermined intervals for 26.5 hours after the intake. The results are shown in Fig. 3. The concentration of BPA in urine reached 90 ng/ml after 30 minutes, then
decreased to 26 ng/ml 60 minutes after intake, and 5 hours after intake, the concentration further decreased to the proximity of the concentration before the intake. BPA was found to be absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine in almost 100% in 24 hours.

**BPA concentrations in urine**

The average BPA concentration in urine samples was 0.81 ng/ml (0.14 – 5.47 ng/ml), and three subjects had a concentration higher than 2 ng/ml. The subject who had the highest concentration, 5.47 ng/ml, had an extremely small amount of urine. Fig. 4 shows an SIM chromatogram of urine sample and Fig. 5 shows a histogram which has a bell shape with the mean almost at the center. The analysis of whole-day urine samples (Table 1) shows that the average concentration of BPA was 0.81 ng/ml (0.24 – 2.03 ng/ml).

![Fig. 1](image1.png)  
**Fig. 1** EI mass spectrum of the BPA derivative. Measurement conditions: column DB-5MS, inner diameter 0.32 μm, length 30 m, film thickness 0.25 μm; column temperature 70°C (2 min) – 20°C/min – 150°C – 10°C/min – 300°C (5 min); carrier gas He at a flow rate of 1 mL/min; ion source temperature 230°C; ionization voltage 70V

![Fig. 2](image2.png)  
**Fig. 2** EI mass spectrum of the $^{13}$C-BPA derivative. Measurement conditions were the same as Fig. 1
the average amount of urine was 2055 ml (1030–3900 ml), and that the average amount of excreted BPA was 1.68 µg (0.48–4.5 µg/day). The amount of excreted BPA was found to be significantly less than the amount we had expected. This may be due to a drop in exposure to BPA achieved by an improvement in inner coatings of cans and the like. The excreted BPA is considered to be derived from food, but the type of food it is derived from remains unknown.

The conjugates of BPA and free BPA are considered to have different endocrine disrupting effects. Thus, determination of free BPA in urine was required. A drink containing 50 µg of BPA-d16 was given to volunteers, and urine samples were collected for 5 hours after intake. The results are shown in Fig. 6. The average total BPA concentration was 57.2 ng/ml (26.5–80 ng/ml), and the average free BPA concentration was 1.13 ng/ml (0.13–5.8 ng/ml). The average amount of excreted BPA was 38 µg (17.6–48.6 µg), which means that 76 % of the given BPA was excreted after 5 hours. The average ratio of free BPA was 2.0 % (0.34–8.1 %). The subjects who showed higher ratios of free BPA may have been affected by their body conditions; in particular, liver function on the day of the experiment. Normal urine samples were also analyzed. The average total BPA concentration was 0.56 ng/ml (0.19–1.38 ng/ml), and the average free BPA concentration was 0.08 ng/ml (0.01–0.27 ng/ml). The average ratio of free BPA was 12 % (2.6

Fig. 3 Relationship between the concentration of the total BPA-d16 in urine and the elapsed time after 100 ml of a drink containing 100 µg of BPA-d16 was consumed

Fig. 4 SIM chromatogram of a real urine sample. Measurement conditions were the same as given in 2.5 GC/MS conditions
CONCLUSION

1. We developed a reliable analytical method for determining trace amounts of BPA in urine.
2. We conducted an excretion experiment by administering 100 μg of BPA-d_{16} to volunteers and found that BPA was absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine, in almost 100% in 24 hours. Thus, the determination of the BPA in whole-day urine samples is considered to enable the estimation of the exposure to

Table 1 Results of whole day urine samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Volume(ml)</th>
<th>BPA(ng/ml)</th>
<th>BPA(μg/day)</th>
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<tr>
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<td>22</td>
<td>3900</td>
<td>0.34</td>
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Ave. 2055±665 0.81±0.49 1.88±1.26
3. The average total BPA concentration in urine samples was 0.82 ng/ml (0.14–5.47 ng/ml, n=91), and the average free BPA concentration was 0.08 ng/ml (0.01–0.27 ng/ml, n=11).

4. From the determination of whole-day urine samples, the exposure to BPA was estimated to be 1.68 μg/day (0.48–4.5 μg/day, n=22) on the average.

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