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# Measurement of bisphenol A in human urine using liquid chromatography with multi-channel coulometric electrochemical detection

Kazuyuki Ouchi[a,b,*], Shaw Watanabe[a]

[a]*Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-0054, Japan*
[b]*MC Medical, Inc., 7-5-25 Nishishinjuku, Shinjuku-ku, Tokyo 160-8355, Japan*

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**Abstract**

Environmental exposure levels of bisphenol A (BPA) in human were investigated by measuring BPA glucuronide (BPA-G) in urine. After enzymatic hydrolysis of glucuronide substances in urine, BPA was extracted with diethyl ether. The extract was analyzed using a column-switching HPLC system employing a $C_8$ and a $C_{18}$ column with multi-channel coulometric electrochemical detection (ECD). The sensitivity and selectivity provided with redox mode of ECD allowed measurement of low level BPA in hydrolyzed urine. The quantification limit of BPA-G in urine was 0.2 ng/ml. RSDs of the intraassay precision were less than 3% and recoveries of the method were over 96% when analyzing BPA spiked urine samples (1.0 and 10 ng/ml). In a group of 48 women students, the level of BPA-G in urine ranged from 0.2 to 19.1 ng/ml with a median concentration of 1.2 ng/ml. Normalized against urinary creatinine, BPA-G ranged from 0.1 to 11.9 ng/mg creatinine with a median of 0.77 ng/mg creatinine.


*Keywords:* Multi-channel coulometric electrochemical detection; Bisphenol A

## 1. Introduction

Bisphenol A (BPA) is one of a number of potential endocrine disruptors which may alter normal hormonal function. BPA is postulated to antagonize the effect of endogenous estrogens and is suspected of being involved in the current increase in the incidence of reproductive malfunction in wild animals and humans [1,2]. BPA is used in the manufacture of polycarbonate plastics and epoxy resins. It is of great concern that these are used as food and beverage containers and as the lining of metal cans, from which BPA leaches into food [3–5]. Thus, humans may routinely ingest trace amounts of BPA. The objective of this study is to investigate the environmental exposure levels of BPA in humans.

The metabolism of BPA has been investigated both in vivo [6,7], and in vitro [8]. The results suggest that BPA absorbed by the intestine is glucuronidated in the liver and excreted as BPA glucuronide (BPA-G) in the urine. Hence, urinary

*Corresponding author. Present address: MC Medical, Inc., 7-5-25 Nishishinjuku, Shinjuku-ku, Tokyo 160-8355, Japan. Tel.: +81-3-5330-7863; fax: +81-3-5330-0499.
*E-mail address:* ouchikazz@ma2.justnet.ne.jp (K. Ouchi).

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BPA-G may be a good marker to estimate exposure levels. GC–MS has been introduced to measure BPA in urine [9]. However, it requires extensive preparative isolation using solid-phase extraction and derivatization prior to analysis, both of which are incompatible with high sample throughput required for routine analysis. HPLC–MS [10,11], HPLC with fluorescence detection [12], and HPLC with coulometric electrochemical detection (oxidation mode) [10,13] are presently used for BPA in serum or plasma, but their applicability to urine samples has not been reported. HPLC–ECD has high sensitivity and selectivity without derivatization. Furthermore, the flow-through porous-carbon graphite coulometric electrodes require no maintenance for extended periods of time, so are suitable for routine analysis [14]. In this study, we evaluated the use of HPLC–ECD for the routine measurement of urinary BPA to: (a) decrease the sample handling required; and (b) increase the selectivity of the electrochemical detection. The use of column-switching and a redox (oxidation/reduction) mode of ECD allowed the routine measurement of trace levels of BPA in urine with simple sample preparation.

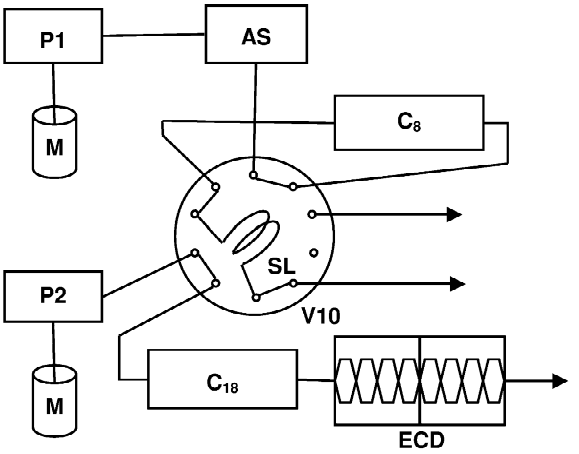


Fig. 1. Column-switching arrangement of the HPLC system used for analysis of BPA in urine. AS, autosampler; $C_8$, $C_8$ column; $C_{18}$, $C_{18}$ column; ECD, eight-channel coulometric electrochemical detector; M, mobile phase; P1 and P2, pumps; SL, 2-ml sample loop; V10, ten-port valve.

## 2. Experimental

### 2.1. Chemicals

Bisphenol A was obtained from Katayama Chemical (Osaka, Japan). Acetonitrile and distilled water (HPLC grade), sodium acetate trihydrate, acetic acid, and diethyl ether were obtained from Kanto Chemical (Tokyo, Japan). β-Glucuronidase (type H-2, from *Helix pomatia*, 10 000 U/ml glucuronidase and 5000 U/ml sulfatase activity) was obtained from Sigma (St. Louis, MO, USA).

### 2.2. Chromatographic conditions

The column-switching HPLC–ECD system (Fig. 1) consisted of two Model 580 pumps (ESA, Chelmsford, MA, USA), a Model 465 autosampler (ESA), a column oven (Bio-Rad, Hercules, CA), a Model C10WE ten-port valve (Valco, Houston, TX, USA), a 2-ml sample loop (Rheodyne, Cotati, CA, USA) and an eight-channel CoulArray Model 5600 electrochemical detector (ESA). The system was controlled and data were acquired and processed using the CoulArray software. The $C_8$ column was a YMC basic 3 μm, 4.6×150 mm (YMC, Kyoto, Japan), and the $C_{18}$ column was an MCM Column 5 μm, 4.6×250 mm (MC Medical, Tokyo, Japan).

The mobile phase for $C_8$ and $C_{18}$ columns consisted of 50 m*M* sodium acetate buffer adjusted to pH 4.8 with acetic acid and acetonitrile (69:31, v/v). The flow-rates through the $C_8$ and $C_{18}$ columns were 0.9 and 1.0 ml/min, respectively. Both columns were maintained at 40 °C.

The total sequence of automated sample analysis was initiated by the autosampler injection. Before starting an analytical cycle, the retention time of BPA on the $C_8$ column ($T_{C8}$: 17.0 min) was measured. The outlet of the $C_8$ column was connected to the CoulArray detector directly, and a standard solution (100 ng/ml) was analyzed. In the analysis sequence, the time of switching the valve was set at $T_{C8}+1.1$ min (18.1 min) to transfer BPA to the 2-ml loop. The band filling the 2-ml loop was transferred to the $C_{18}$ column by switching the valve, and the $C_8$ column was then back-flushed. BPA was eluted from the $C_{18}$ column at 46.2 min after the autosampler

injection. After the back-flush of the $C_8$ column was completed by switching the valve at 55 min, the system initiated the next cycle.

### 2.3. Coulometric array detector conditions

The potentials of the eight coulometric electrodes in the array (E1–E8) were optimized for selective measurement of BPA on the basis of its voltammetric behavior [15]. The current–voltage curve for BPA under the chromatographic condition is presented in Fig. 2. The potentials of the first four electrodes (E1–E4) were set at 0, 320, 540 and 620 mV, respectively. The following four electrodes (E5–E8) were set across the reduction potential region to detect BPA by reducing the oxidized BPA generated by the upstream electrodes. The potentials of the electrodes were set at 120, 0, −50 and −100 mV, respectively. BPA was detected at E3 and E4 in oxidation mode, and at E5 and E6 in redox mode. Quantification of BPA was performed at E6 with the average peak height of bracketing external standards included with every ten samples. The response ratios of E6/E5 were used as a qualitative assurance measure for the peak purity of BPA [16,17].

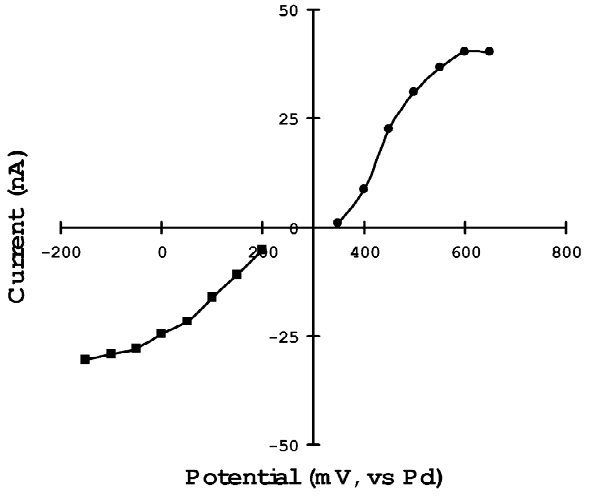


Fig. 2. Current–voltage curve for BPA showing cumulative peak heights as a function of the potential applied to the coulometric electrodes under oxidation mode (●) and redox mode (■).

### 2.4. Sample preparation

Free BPA and BPA-G levels were measured in first void (morning) urine samples obtained from 48 women students. Samples for the HPLC were prepared as described below.

#### 2.4.1. Determination of free BPA levels

A 0.5-ml volume of urine was vortex-mixed for 10 s with 1 ml of diethyl ether. Following centrifugation (3000 *g* for 5 min), the ether phase (upper layer) was transferred into another test tube and the water phase (lower layer) was extracted with 1 ml of diethyl ether in the same manner. The combined ether phases were dried under nitrogen flow at 40 °C. The remaining residue was dissolved in 100 μl of 30% acetonitrile by vortex-mixing for 10 s. A 50-μl volume was then analyzed.

#### 2.4.2. Determination of total (conjugated and free) BPA levels

A 0.5-ml volume of urine was mixed with 20 μl of β-glucronidase and 0.5 ml of buffer (0.1 *M* sodium acetate, pH 5.0 containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA). This mixture was incubated at 37 °C for 3 h. It was then vortex-mixed for 10 s with 1 ml of diethyl ether. Following centrifugation (3000 *g* for 5 min), the ether phase was transferred into a test tube and the water phase was extracted with an additional 1 ml of diethyl ether in the same manner. Gelation of the ether phase was sometimes found after centrifugation in the second extraction. In this case, the tube was shaken gently to aggregate the gel and centrifuged again. The combined ether phase was dried and the residue was dissolved as above and a 50-μl volume was analyzed as before.

### 2.5. Evaluation of limits of detection and linearity

Stock standards of BPA (0.1 mg/ml) were made in acetonitrile and stored at −30 °C. Working standard solutions were prepared by dilution of stock standard in 30% acetonitrile (v/v). Linearity and sensitivity were evaluating using 50-μl injections of 1-, 10-, 100- and 1000-ng/ml standards in triplicate.

### 2.6. Precision of the method

Precision of the method was investigated by analyzing spiked urine samples. They were prepared by adding 50 μl of 10- or 100-ng/ml standards to 0.5 ml of the urine pool sample, which was prepared by combining five urine samples (ranging in concentration of BPA-G from less than 0.3 ng/ml among the 48 samples). Another 0.5 ml of the same urine sample pool had 50 μl of 30% acetonitrile added and served as the baseline (control) value. Four spiked urine samples at each BPA level were prepared as described above for free and total BPA levels in urine. Intraasay precision was calculated as the percentage relative standard deviation (RSD) from the four replicate analysis in a series of routine measurements.

## 3. Results

BPA in urine was successfully measured at E6 using the redox mode of detection. Detection of BPA at E3 with oxidation mode was problematic due to the co-elution of endogenous substances existing in the urine. These interferences, however, were not detected at E5–E7 under redox mode because some of them were electrochemically irreversible while others needed lower potential than BPA for reduction. The typical chromatograms obtained on the E5 and E6 sensors for enzymatically hydrolyzed urine samples are presented in Fig. 3. Due to the column switching technique and selectivity of the redox mode, it was unusual to observe more than three other resolved peaks in the chromatograms on sensors E5 and E6. The peak height ratio between E5 and E6 obtained from a pure standard matched the peak in urine samples, thereby assuring that the peak was separated from potential interferences that could elute coincidentally at a similar retention time. When the acetonitrile content of the mobile phase was increased to more than 31% in order to decrease the analysis time, the quantification was compromised as the peak eluting immediately prior to BPA shifted so that it now interfered with this analyte. On the other hand, when determining free BPA levels (i.e. without enzymatic hydrolysis), no peaks were observed around the retention time of BPA in the chromatograms on sensors E5 and E6 so that it was possible to increase the acetonitrile content of mobile phase to shorten the analysis time.

The on-column sensitivity of the HPLC system under redox mode of detection was 50 pg of BPA (signal-to-noise ratio of 3:1). As the urine samples were concentrated to one-fifth of their original volume in the sample preparation procedures, the

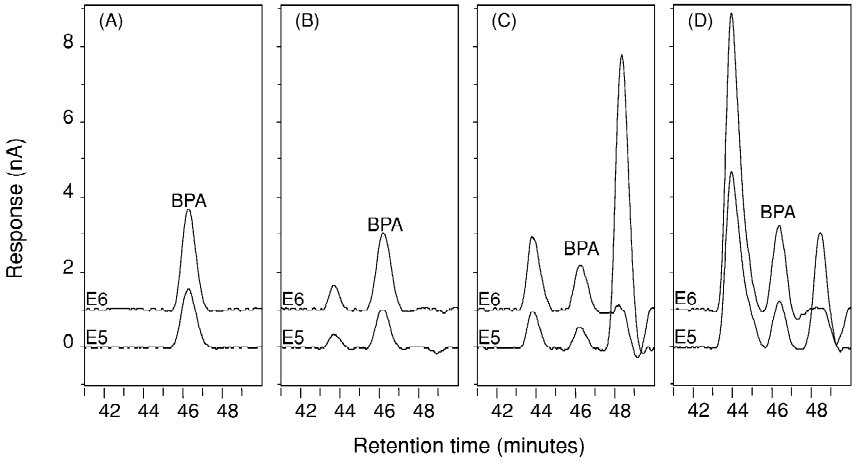


Fig. 3. Two-channel chromatograms of a BPA standard (500 pg on column) (A) and three enzymatic hydrolyzed urine samples (B–D). The potentials of E5 and E6 were 120 and 0 mV (vs. Pd), respectively. The chromatogram patterns of the 48 hydrolyzed samples tended to fall into three groups (B–D) depending upon the adjacent peaks. Qualitative values (ng/ml) and response ratios [(sample response ratio)/(standard response ratio)] obtained for the samples were: (B) 1.54 ng/ml, 1.13; (C) 0.91 ng/ml, 0.84; and (D) 1.79 ng/ml, 0.92.

Table 1
Precision of the method ($n$=4)

| | Mean±%RSD, ng/ml | |
|---|---|---|
| | Free BPA protocol | Total BPA protocol |
| Urine pool | n.d. | 0.26±12 |
| 1 ng/ml spike in urine | 1.03±2.7 | 1.22±2.6 (0.96[a]) |
| 10 ng/ml spike in urine | 10.36±1.8 | 10.11±1.2 (9.85[a]) |

[a] Background BPA-G level in urine pool (0.26 ng/ml) was subtracted out of the spiked sample levels ($n$=4).

limit of detection of BPA in urine was 0.2 ng/ml for a 50-μl volume analyzed on the HPLC system. By least-squares regression analysis, detector response was directly proportional to standard concentration, and calibration curve was linear from 50 pg to 50 ng on column with a correlation coefficient ($r$) of 0.9998. Table 1 presents the precision of the method obtained by measuring a urine pool sample and two levels of spiked urine samples in four repetitive injections. Analytical recovery estimated by analyzing spiked urine samples was 103% for the determination of free BPA level. This decreased to 96% under conditions where gelation of the ether phase was observed for the second extraction. The RSDs of the method in four repetitive injections were less than ±3% with 1 and 10 ng/ml spiked urine samples, and ±12% for with urine pool sample containing BPA close to the limit of detection (0.26 ng/ml).

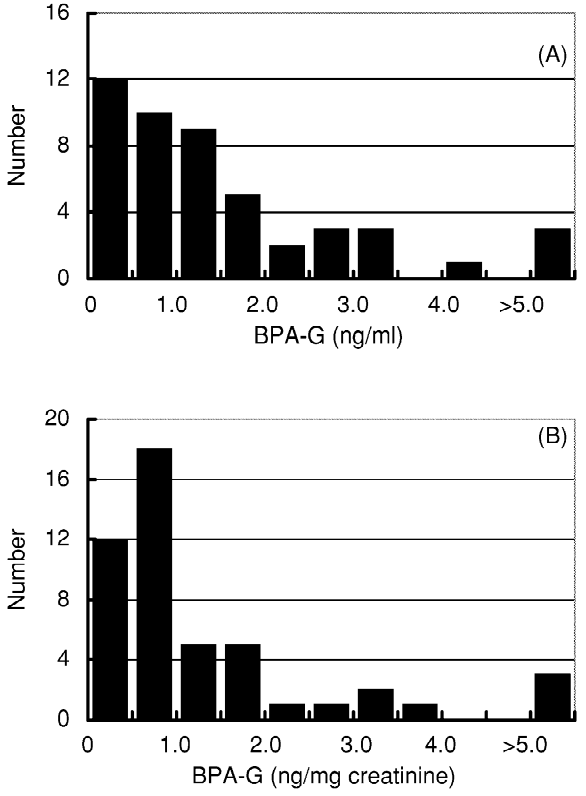


Fig. 4. Frequency distribution of urinary BPA-G levels in 48 women students. (A) Urinary BPA-G levels; (B) normalized BPA-G against urinary creatinine.

The levels of free BPA and BPA-G in urine of 48 women college students were studied. Free BPA levels in urine were below the detection limit (0.2 ng/ml) except for one sample where the level was 0.2 ng/ml. BPA-G was detected in all samples with distribution of 0.2–19.1 ng/ml (median 1.2 ng/ml). Normalized against urinary creatinine, it ranged from 0.1 to 11.9 ng/mg creatinine (median 0.77 ng/mg creatinine). The BPA-G level of the sample in which free BPA was detected was 19.1 ng/ml which was the highest level in the 48 samples. The frequency of distribution of the urinary BPA-G levels and the normalized BPA-G levels against urinary creatinine are shown in Fig. 4.

## 4. Discussion

Several modes of detection are available with multi-channel coulometric ECD [15]. Oxidation mode is the first choice for analysis of phenolic compounds, where analytes are detected by oxidation at the electrodes corresponding to their oxidation potential [16]. If analytes are electrochemically reversible, redox mode is usually more selective. In this mode oxidation potentials of the analytes are applied for the first several electrodes followed by incrementally decreasing potentials for the remaining

electrodes. The oxidized analytes initially generated at the upstream electrodes are then reduced at the downstream electrodes. For the analysis of BPA in hydrolyzed urine, we found that the interferences in the oxidation mode were effectively removed in redox mode. The relatively high reduction potential of BPA in redox mode (0 mV vs. Pd) allowed selective and sensitive detection. The interferences in oxidation mode had higher reduction potentials than BPA, so consequently, they did not disturb detection of BPA in redox mode. The column-switching protocol minimized interferences from other endogenous electroactive compounds existing in the urine. Furthermore, the back-flush of the first column prevented contamination of late-eluting compounds.

The exposure level of BPA in human was investigated by measuring BPA-G in urine. The metabolism of BPA has been well characterized in the rat with the major metabolite being BPA-G. In adult female rats after oral administration of [$^{14}$C]BPA (100 mg/kg), most of the label was found in urine and feces (>91%) with a small amount in the carcass (~1%) after 6 days. Urinary excretion of $^{14}$C was 21–42% (dependent upon strain) of the original dose, and BPA-G was the major metabolite in urine (>81% of $^{14}$C) [7]. BPA-G was detected in all urine samples of 48 women students ranging from 0.1 to 11.9 ng/mg creatinine (median 0.77 ng/mg creatinine). Although less is known regarding BPA metabolism in humans, the exposure level of the group is estimated to be from 0.6 to 71.4 μg/day assuming that 20% of the ingested BPA is excreted in urine and the amount of creatinine excreted in urine is 1.2 g/day. The estimated exposure levels of BPA of our analysis are reconciled with the exposure levels speculated from data on the amount of BPA leached from food cans (maximum 23 μg/can) [3]. BPA-G in urine may be a good marker to evaluate exposure levels of BPA for humans. The use of this method will now enable better investigation into the relationship between lifestyle and the levels of BPA to which humans are exposed.

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