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Size-exclusion flow extraction of bisphenol A in human urine for liquid chromatography–mass spectrometry

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Abstract

We report an approach for assessing human exposure to bisphenol A (BPA), which involves measuring the glucuronide in urine sample that were subjected to a novel size-exclusion flow extraction method. The present approach includes the addition of $^{13}C_{12}$-BPA, enzymatic deconjugation, and the proposed sample preparation method. The sample solution is separated and detected by liquid chromatography–mass spectrometry (LC–MS). The following are used for the LC–MS: a reversed-phase separation column, electrospray ionization (ESI), negative mode, and single ion monitoring (SIM) with *m/z* 227 for BPA and *m/z* 239 for $^{13}C_{12}$-BPA. The detection limit was 0.1 ng ml^{-1} and the calibration curves (0.45–90 ng ml^{-1}) had correlation coefficients exceeding 0.999. To urine samples requiring deglucuronidation, β-glucuronidase was added followed by incubation at 37 °C for 3 h. After the enzymatic treatment, the samples were subjected to the extraction in the reversed-phase (ODS) and size-exclusion (GPC) modes. It was possible to extract, clean up and concentrate BPA in a single run of 20 min by means of the novel extraction method. The method enables the determination of standards and may be applied to the detection of trace amounts of BPA in human urine samples.

Keywords: Sample preparation; Bisphenol A

1. Introduction

The report entitled "Global Assessment of the State-of-the-Science of Endocrine Disruptors", is a result of a comprehensive review of publicly available scientific literature on endocrine disruptors organized by the International Programme on Chemical Safety [1]. In Japan, the Health Sciences Research conducted by the Ministry of Health, Labour and Welfare indicated that risk assessment, human exposure and toxicological studies have been conducted and analytical techniques for endocrine disruptors have been provided by many researchers [2]. Bisphenol A (BPA) contributes significantly to the environmental problem as evidenced by a number of studies indicating the effects of BPA in the "low dose" range, including its effects at the ppt level, in the last two years [3–6]. Thus, the potential effects of BPA on human health and human exposure to BPA must be examined for risk assessment.

The metabolism and kinetics of low doses of BPA in human following oral administration have been the subject of numerous studies. The efficient glucuronidation of BPA and the rapid excretion of the formed glucuronide were reported in human [7]. In addition, it was reported that BPA was glucuronidated in liver microsomes during metabolisation [8]. Therefore, we assessed BPA levels in human plasma samples by monitoring both free and glucuronidated BPAs [9]. In preparation for a population-based human exposure study, we reviewed recent literature on the current methods for analyzing BPA in human samples (Table 1). In general, the gas chromatography–mass spectrometry (GC–MS) method is the most commonly employed technique for the environmental analysis of endocrine disruptors. However, due to the low volatility of some compounds including a hydroxyl group, derivatization steps aimed at producing more BPA are required to improve the sensitivity of the subsequent GC–MS analysis. Therefore, the use of chemical derivatives of improve analysis has been a practice in the GC–MS analysis of a trace amount of BPA in water [36]. On the other hand, liquid chromatography (LC) and enzyme-linked immunosorbent assay (ELISA) are used for the determination

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Table 1
The recent literature for current methods for analyzing and assessment of BPA in human biological samples

Target BPA form	Biological sample	Analytical technique	Sample preparation	LOD ($ng\,ml^{-1}$)	LOQ ($ng\,ml^{-1}$)	Ref.
Free	Serum	LC–MS, LC–ED	SPE	0.1–0.2	–	Sajiki et al. (1999) [10]
Free	Serum	LC–ED	SPE	0.01	0.05	Inoue et al. (2000) [11]
Free	Serum	LC–FD (using labeling reagent)	Column-switching	0.04	0.1	Kuroda et al. (2003) [12]
Free	Serum	ELISA, GC–MS (derivatization)	Proteins precipitation	–	0.3	Ohkuma et al. (2002) [13]
Free	Serum	GC–MS (derivatization)	SPE	0.005	0.01	Yoshimura et al. (2002) [14]
Free	Serum	ELISA	SPE	0.2	–	Yamada et al. (2002) [15]
Free	Serum	Novel ELISA	SPE	–	–	Takeuchi et al. (2002) [16]
Free	Serum, Urine	GC–MS (derivatization)	Proteins precipitation	5.0–10	10	Kudo et al. (1998) [17]
Free	Plasma	LC–FD (using labeling reagent)	SPE	0.05	1.0	Watanabe et al. (2001) [18]
Free	Red blood cell	LC–ED	novel SPE	–	–	Sajiki (2003) [19]
Free	Semen	LC–MS, ELISA	SPE	0.1	0.5	Inoue et al. (2002) [20]
Free	Saliva	GC–MS	–	3	15	Zafra et al. (2002) [21]
Free	Saliva	GC–MS (derivatization), HPLC	Proteins precipitation	–	1.0	Atkinson et al. (2002) [22]
Free	Saliva	LC–UV	Proteins precipitation	0.1	0.3	Arenholt-Bindslev et al. (1999) [23]
Free	Tissue	LC–UV	Proteins precipitation	25	–	Csanady et al. (2002) [24]
Free and glucuronidated	Plasma	LC–MS	Novel SPE	0.1	1.0	Inoue et al. (2001) [9]
Free and glucuronidated	Urine	GC–MS (derivatization)	SPE	0.12	0.27	Brock et al. (2001) [25]
Free and glucuronidated	Urine	LC–ED	Column-switching	–	0.2	Ouchi et al. (2002) [26]
Free and glucuronidated	Urine	LC–ED	Proteins precipitation	0.25	–	Hanaoka et al. (2002) [27]
Free and glucuronidated	Urine	GC–MS (derivatization)	SPE	–	0.1	Tsukioka et al. (2003) [30]

Abbreviations: LOD, limit of detection; LOQ, limit of quantitation; LC–MS, liquid chromatography–mass spectrometry; LC–ED, liquid chromatography–electrochemical detection; LC–FD, liquid chromatography–fluorescence detection; ELISA, enzyme linked immuno-sorbent assay; GC–MS, gas chromatography–mass spectrometry; SPE solid phase extraction.

of trace amounts of BPA in human samples (Table 1). Our studies reported that sufficiently sensitive LC method for the determination of BPA in biological sample was developed by the derivatization [12,18]. In addition, we found that the ELISA method may give erroneous values due to non-specific binding to the antibody, leading to an overestimation of trace amounts of BPA in human semen samples [20]. Therefore, it may lack precision for the determination of trace levels of BPA in human samples. On the other hand, for the detection of trace levels of BPA in human samples by LC–electrochemical detection (LC–ED) and fluorescence detection (FD) methods more selective and accurate information is needed other than peak response and retention time [11,12,18,19,26,27]. Here, we demonstrate that the liquid chromatography–mass spectrometry (LC–MS) technique using a labeled surrogate standard is the method of choice for the simple, accurate and selective analysis of BPA levels in human samples [9,20].

Recently, the leaching of BPA from food-contacting plastics and can coatings, and the presence of BPA in foods has been reported [28–32]. Therefore, it is possible that humans are exposed to BPA via a variety of daily activities. In human exposure studies, trace levels of BPA were found in human blood samples. However, compared to urine collection, blood collection is thought to be unsuitable for exposure assessment of healthy people because of its invasiveness. Equally important in measuring BPA in human samples are the following: its contamination in the laboratory and during pretreatment, and the monitoring of its free forms and metabolites. Brock et al. reported the use of the GC–MS method after the enzymatic deglucuronidation step to measure BPA levels in human urine samples in order to obtain a reference range [25]. In the present study, we used human urine samples for the assessment of human exposure to BPA as well.

In the preliminary study for assessment and determination of BPA in human samples, the sample preparation method is selected. Solvent evaporation, steam distillation and liquid–liquid extraction methods, which were used in the past, have been replaced by the more efficient and versatile solid-phase extraction (SPE) technique for biological samples. The preparation of human samples containing BPA using SPE has been reported (Table 1). In our practical study, the SPE technique was used for the preparation of BPA in human samples. However, this technique presented several problems. First, there was contamination of trace levels of BPA during sample preparation with SPE [11]. Second, human samples are at a high risk of infection by HIV, hepatitis, or other unknown diseases or viruses. Therefore, manual handling of biological samples directly by laboratory researchers during sample preparation should be avoided as much as possible. As a solution, the direct injection of sample into a system consisting of a reversed phase and a size exclusion column which were connected on-line was developed for the extraction and fraction of BPA in human urine samples.

In the present study, an accurate, safe, sensitive, selective and high-throughput analytical method was developed for the quantification of BPA by using stable isotopically labeled internal standards. The use of β-glucuronidase to hydrolyze glucuronide metabolites enables the quantification of both free and glucuronidated forms of BPA. We evaluated the method for screening these compounds in human urine samples with creatinine correction.

2. Experimental

2.1. Reagents

Bisphenol A (BPA) standard was purchased from Kanto Chemical Industries, Ltd. (Tokyo, Japan). $^{13}C_{12}$-BPA surrogate standard was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). HPLC-grade acetonitrile for the mobile phase was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), as were all pesticide-grade solvents used in sample preparation. Purified water was obtained using a Milli-Q gradient A10 Elix with an EDS polisher system (Millipore, Bedford, MA., USA). The EDS polisher is a new filter purchased from Millipore, Japan. The water refined by this filter was non-contamination of BPA ($<0.01\,ng\,ml^{-1}$) [33]. Creatinine determination was carried out using the Wako creatinine test (Law of Jaffé) from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

2.2. Urine samples

Urine samples were obtained from four healthy volunteers aged 21–28 years. All samples were stored at −80 °C prior to use.

2.3. Liquid chromatography–mass spectrometry (LC–MS) measurement

Liquid chromatography with electrospray mass spectrometry (LC–MS) was performed using an Agilent 1100 MSD-SL system (Agilent Technologies, Palo Alto, USA). The injection volume was 5.0 μl in the needle washing mode. The column used was Mightysil RP-18 GP Aqua (150 mm × 2.0 mm, particle size 5 μm) with a Mightysil RP-18 GP pre-column (20 mm × 2.0 mm, particle size 5 μm). The column oven was maintained at 40 °C for the separation of the compounds. The separation was carried out using a mobile phase of 0.01% acetic acid in water and acetonitrile (65/35 (v/v)) at a flow rate of $0.2\,ml\,min^{-1}$.

The working conditions for the electrospray MS were as follows: the drying nitrogen gas was set at a temperature of 350 °C and was introduced into the capillary region at a flow rate of $12\,l\,min^{-1}$; the capillary was held at a potential of 3500 V relative to the counter electrode in the negative ion mode. The fragmentor voltage was 140 V during the chromatographic run. When working in the selected ion

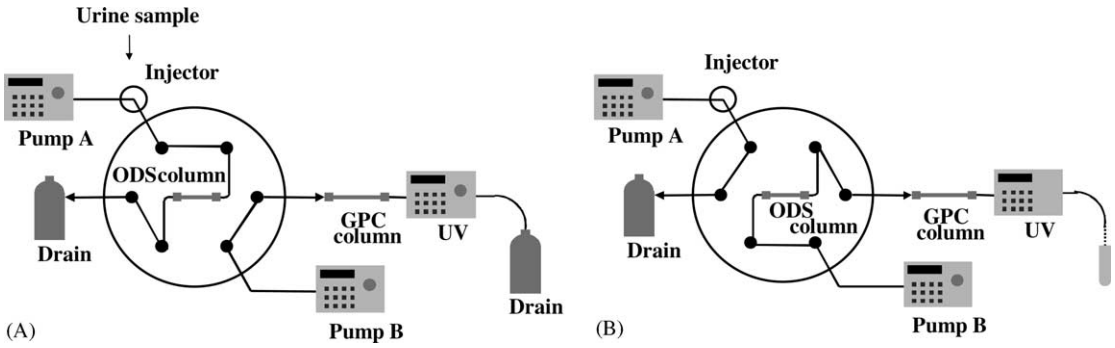

Fig. 1. Schematic representation of the column-switching extraction system. (A) A urine sample was directly injected onto the TSK–precolumn BSA–ODS/S in stream of pure water using pump A. (B) This configuration allowed to back-flashing the ODS column to the GPC column and the detector in the flow path of pump B.

monitoring (SIM) mode, the *m/z* 227 and 239 ions, were assigned as the $[M–H]^-$ of BPA and $^{13}C_{12}$-BPA, respectively.

Standard solutions of BPA were prepared in methanol and added to a fixed concentration of $^{13}C_{12}$-BPA. Quantitative analysis was performed using SIM in order to maximize sensitivity. This resulted in a calibration curve covering the concentration range of 0.45–90 ng ml^{-1}. BPA concentrations were calculated relative to $^{13}C_{12}$-BPA standards that were added to the samples prior to extraction to give a final extract concentration of 2.0 ng ml^{-1} in urine samples. Eight-point calibrations were performed daily for all analyzers with internal standards. The detection limit (DL) was calculated as three times the standard deviation of the analyte concentration determined in the spiked blank sample.

2.4. Enzymatic deconjugation of glucuronidated form of BPA in urine

One milliliter of human urine sample was buffered with ammonium acetate (100 μl, 1.0 M, pH 6.8). After *E. coli* β-glucuronidase (10 μl, 89 U ml^{-1}; Fluka Chemie AG, Buchs, Switzerland) was added, the sample was sealed in a glass tube and gently mixed. Quantitative glucuronidase hydrolysis to obtain the free BPA was accomplished by incubating at 37 °C for 3 h. After the enzymatic deconjugation, the sample solution was centrifuged at 3000 rpm for 15 min to remove proteins. This treatment is sufficient to deconjugate the glucuronidated BPA [25]. The sample was subjected to the proposed sample preparation method.

2.5. Novel sample preparation for LC–MS measurement of BPA in urine samples

A previously reported method for the sample preparation of BPA in human plasma was modified [14].

The column-switching extraction system consists of two LC pumps (Shimadzu LC-10AS (pump A) and LC-10ADvp (pump B) pumps: Shimadzu, Kyoto, Japan), a detector (SPD-10A, UV 225 nm [11], Shimadzu, Kyoto, Japan) and extraction columns (TSK-PRECOLUMN BSA-ODS/S 4.6 mm × 10 mm: 5 μm, TOSOH Co., Tokyo, Japan, and CLNpak PAE-800 8.0 mm × 300 mm Shodex Co., Tokyo, Japan). The column-switching extraction system, as depicted in Fig. 1, was used for the direct injection of urine samples. A urine sample (0.5 ml) was directly injected onto the TSK–precolumn BSA–ODS/S in stream of pure water at a rate of 1.0 ml min^{-1} using pump A for 5 min (Fig. 1A). While the effluent of this column was directed to drain during the 5 min lading period, the sample was extracted on the ODS column. After 5 min, the position of the switching valve was changed (Fig. 1B). This configuration allowed to back-flashing the ODS column to the GPC column and the detector in the flow path of pump B. The actual GPC separation and clean up were carried out using an acetonitrile solution at a flow rate of 1.0 ml min^{-1}. Upon detection of BPA, the fraction was collected for 1 min. The run time for the preparation of the sample mixture was 20 min. The solution was dried under a stream of nitrogen at 40 °C prior to re-suspension in 50 μl of methanol. The sample was then subjected to LC–MS as described below.

3. Results and discussion

3.1. LC–MS validation for determination of BPA

In the analysis using electrospray MS with flow injection analysis of BPA standard solutions, the *m/z* 227 ion, which was assigned the $[M–H]^-$ ion, was observed as the main peak. In addition, a fragment ion at *m/z* 212, resulting from a cleavage of one of the CH_3 groups is observed [9]. We reported that the ion at *m/z* 227 can be used in the negative ion mode to both screen for and quantitatively analyze BPA in standard samples [9,20].

The most important parameters affecting LC–MS are the fragmentor voltage and the composition of mobile phase. In order to establish the optimum fragmentor voltage for the detection of BPA, the *m/z* 227 signal for BPA versus

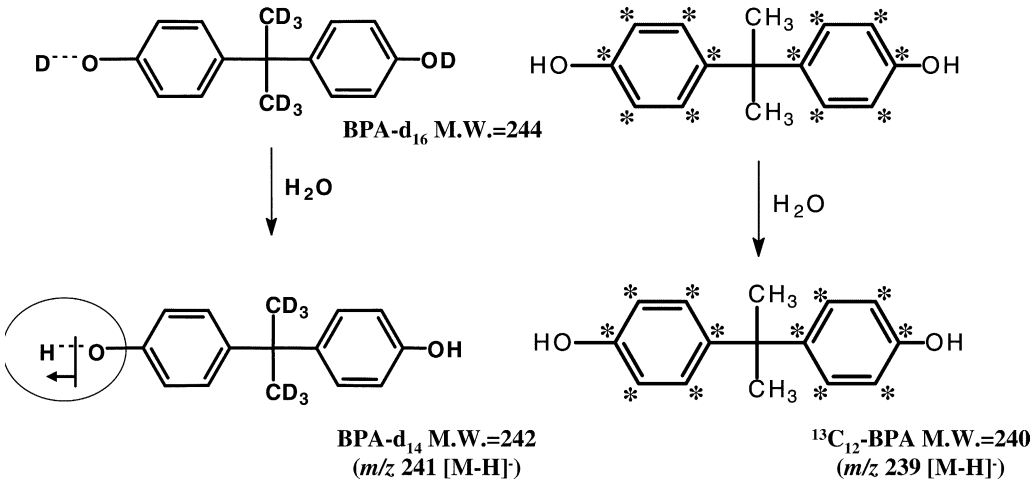

Fig. 2. Structures of stable isotopically labeled internal standards (BPA-d_{16} and $^{13}C_{12}$-BPA) and reaction with water.

fragmentor voltage was investigated. The monitored signal showed a maximum in 0.01% acetic acid at 140 V for BPA [9,20].

3.2. Analytical method parameter and calibration for determination of BPA

In general, it is very important that LC–MS determination of a target compound be accomplished by using stable isotopically labeled internal standards. When working in the SIM mode, the $[M–H]^-$ ion of BPA was assigned to the signal at *m*/*z* 227. On the other hand, stable isotopically labeled internal standards of BPA, such as BPA-d_{16} and $^{13}C_{12}$-BPA, were *m*/*z* 241 and 239, respectively (Fig. 2). BPA-d_{16} (MW 244) was transformed into BPA-d_{14} (MW 242) in water (Fig. 2). The two acid deuterium atoms of BPA-d_{16} are immediately exchanged against protons when dissolved in a protic medium. It is for the reason that *m*/*z* 241 was assigned as $[M$ (BPA-d_{14})$–H]^-$ in the negative ion mode. The deuterium atoms bound to carbon atoms are of course stable. The phenolic deuterium atoms, however, are due to their acidity easily exchangeable. They would not only exchange in water, but also in any other protic solvent and even in aprotic solvents that inevitably contain traces of water. This, however, does not impair the utility of BPA-d_{14} as isotopically labeled internal standard. In the present study, therefore, we used a more stable internal standard, $^{13}C_{12}$-BPA, rather than BPA-d_{16}. The $^{13}C_{12}$-BPA standard is not transformed and its $[M–H]^-$ signal is assigned to *m*/*z* 239.

The calculated detection limit was 0.1 ng ml^{-1} for LC–MS. In addition, the limit of quantitation calculated when the signal-to-noise ratio was higher than 20 was 0.1 ng ml^{-1} in urine samples. LC–MS with SIM calibration was used to investigate the linearity of the method. Peak area ratios with respect to $^{13}C_{12}$-BPA internal standard were plotted. The response was found to be linear in the validated range with "area ratio = 0.025 × amount ratio + 0.03 (correlation: 0.999)". The method may be applied to the detection of trace amounts of BPA in human urine samples.

3.3. Optimal conditions and recovery of BPA from the urine samples subjected to the sample preparation method

The novel sample pre-treatment method was carried out by using a reversed-phase ODS column and a size-exclusion GPC column for extraction. The effluent was fractionated; then, the fractions were collected and measured by UV (225 nm) detector and the off-line LC–MS system. Based on the results of the detection of BPA standard solution in each fraction, we decided to use the elution period (15–16 min for 1.0 min) during which most of the BPA could be isolated in the largest amount (Table 2). The fraction was evaporated to dryness, adjusted with methanol (50 μl) and examined for their recoveries and relative standard deviations (R.S.D.s). We found that using this method, the recoveries and R.S.D.s of BPA at 1.0 and 5.0 ng ml^{-1} in human urine samples were 107.0% (R.S.D.: 7.4%, $n = 6$) and 98.2% (R.S.D.: 4.1%, $n = 6$). The chromatograms of BPA (SIM: 227) and $^{13}C_{12}$-BPA (SIM: 239) from the recovery test (5.0 ng ml^{-1}

Table 2
Relationship between BPA detection levels and fraction elution time by the proposed extraction system

Fraction time (min)	Relative peak area of BPA (%)
10–11	0
11–12	0
12–13	0
13–14	0
14–15	0
15–16	95.0
16–17	3.52
17–18	1.23
18–19	0.15
19–20	0.10

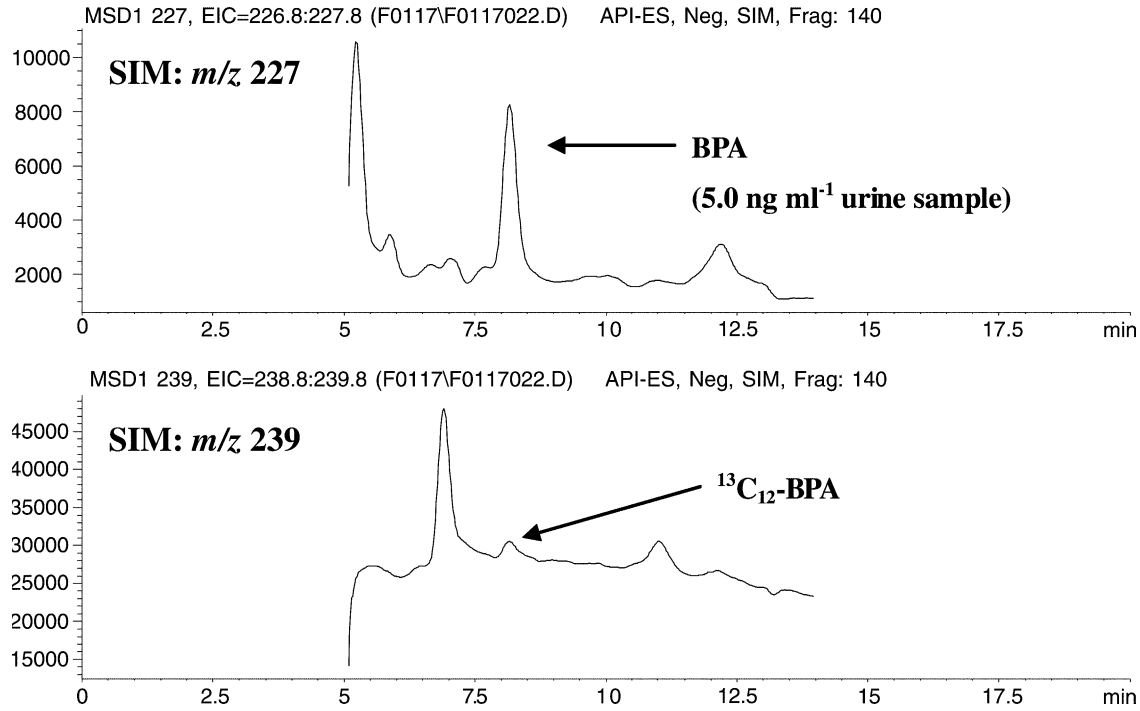

Fig. 3. Chromatograms of BPA and internal standard ($^{13}C_{12}$-BPA) in human urine sample (recovery test: added 5.0 ng ml^{-1} in urine).

Table 3
BPA concentration in human urine samples after meals (2 and 5 h) used in the present method

	Age		2 h			5 h		
			Creatinine (mg dl^{-1})	BPA (ng ml^{-1})		Creatinine (mg dl^{-1})	BPA (ng ml^{-1})	
				(−) β-g1ucuronidase	(+) β-g1ucuronidase		(−) β-g1ucuronidase	(+) β-g1ucuronidase
A	28	Male	21.5	ND	ND	7.0	ND	ND
B	25	Female	5.5	ND	ND	6.7	ND	ND
C	23	Male	22.1	ND	ND	19.1	ND	0.26
D	21	Female	9.0	ND	ND	13.9	ND	0.38

ND <0.1 ng ml^{-1}.

in urine sample) are shown in Fig. 3. Accuracy of the method was expressed as recovery relative to the internal standard.

3.4. Measurement of BPA in human urine samples

It was determined previously that the limit of quantitation was 0.1 ng ml^{-1} at signal-to-noise ratios higher than 20 in human urine samples. Healthy volunteers were enrolled in this study. The aim of the present study was to investigate urinary BPA levels in human after ingestion of canned foods. The experimental design was as follows: four volunteers had consumed the same meal and drinks (green tea in PET bottle): in addition, two volunteers (C and D) had consumed the canned foods. Then, we examined four urine samples for the presence of BPA after meals (2 and 5 h) using the present method. As expected, the average urinary creatinine level was 13.1 ± 6.1 mg dl^{-1} (Table 3). All urine samples analyzed by the LC–MS system after being subjected to the novel sample preparation method demonstrated no detectable free BPA (not detected: ND <0.1 ng ml^{-1}) before deglucuronidation. After deglucuronidation, trace amounts of urinary BPA in healthy volunteers (C and D) were detected after 5 h (Table 3).

Recently, there have been reports on the contamination and leaching of BPA from food cans. It is, however, difficult to say that main source of human exposure is the ingestion of food contaminated by BPA leaching from cans. We confirmed that there is a need for investigation and monitoring of human exposure of BPA on a large scale.

4. Conclusions

Our findings suggest that the LC–MS method can detect the presence of BPA at very low levels (the limit of quantitation, 0.1 ng ml^{-1}) in human urine samples. In addition,

we have demonstrated that other sources of human exposure to BPA may also exist such as drinking water, indoor air, medical treatment, work environment, etc. [13,27,33–35]. On the other hands, the presence of BPA at very low levels in human urine may undergo a changed by human living factors. Therefore, the development of analytical techniques for quantification and survey of the presence of BPA in human sample need to be careful and control about the effects of sampling time, taking foods, medical treatment, work environment, etc.

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