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Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection

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Abstract

A simple and sensitive method using high-performance liquid chromatography with multi-electrode electrochemical detection (HPLC–ED) including a coulometric array of four electrochemical sensors has been developed for the determination of bisphenol A in water and human serum. For good separation and detection of bisphenol A, a CAPCELL PAK UG 120 C_{18} reversed-phase column and a mobile phase consisting of 0.3% phosphoric acid–acetonitrile (60:40) were used. The detection limit obtained by the HPLC–ED method was 0.01 ng/ml (0.5 pg), which was more than 3000-times higher than the detection limit obtained by the ultraviolet (UV) method, and more than 200-times higher than the detection limit obtained by the fluorescence (FL) method. Bisphenol A in water and serum samples was pretreated by solid-phase extraction (SPE) after removing possible contamination derived from a plastic SPE cartridges and water used for the pretreatment. A trace amount (ND~0.013 ng/ml) of bisphenol A was detected from the parts of cartridges (filtration column, sorbent bed and frits) by extraction with methanol, and it was completely removed by washing with at least 15 ml of methanol in the operation process. The concentrations of bisphenol A in tap water and Milli-Q-purified water were found to be 0.01 and 0.02 ng/ml, respectively. For that reason, bisphenol A-free water was made to trap bisphenol A in water using an Empore disk. In every pretreatment, SPE methods using bisphenol A-free water and washing with 15 ml of methanol were done in water and serum samples. The yields obtained from the recovery tests using water to which 0.5 or 0.05 ng/ml of bisphenol A was added were 83.8 to 98.2%, and the RSDs were 3.4 to 6.1%, respectively. The yields obtained from the recovery tests by OASIS HLB using serum to which 1.0 ng/ml or 0.1 ng/ml of bisphenol A was added were 79.0% and 87.3%, and the RSDs were 5.1% and 13.5%, respectively. The limits of quantification in water and serum sample were 0.01 ng/ml and 0.05 ng/ml, respectively. The method was applied to the determination of bisphenol A in healthy human serum sample, and the obtained detection was 0.32 ng/ml. From these results, the HPLC–ED method should be the most useful in the determination of bisphenol A at low concentration levels in water and biological samples.

Keywords: Bisphenol A

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1. Introduction

In 1993, bisphenol A, 4,4′-(1-methylethylethylidene) bisphenol (Fig. 1), was reported to have a weak estrogenic activity [1]. More recently, Gaido et al. reported that estrogenicity of bisphenol A in vitro was observed to be 15 000-times less active than 17β-estradiol [2]. Whereas, Nagel et al. [3] and Vom Saal et al. [4] reported the presence of reproductive or developmental effects following the administration of low oral doses of bisphenol A. However, bisphenol A is an important chemical used principally as a monomer in the manufacture of a multitude of chemical products including epoxy resins and polycarbonate. It is used extensively in the industrialized world and is present in a diverse range of manufactured articles. Accordingly, it is highly possible that bisphenol A leaches into the environment as well as into human bodies. Thus, it is desirable to develop an easy and sensitive analytical method for the determination of a trace amount of bisphenol A.

There are many analytical methods for the determination of bisphenol A in environmental water, air and plastic waste including gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) equipped with ultraviolet (UV) or fluorescence (FL) detection systems [5–9]. In addition, it was reported that Gonzalez-Casado et al. developed a sensitive method for the determination of bisphenol A in water by formation of trimethylsilyl derivatives from bisphenol A followed by GC–MS [10]. However, there are only a few easy and sensitive methods for the determination of a trace amount of bisphenol A in water and biological samples. Therefore, we examined to develop a simple and sensitive HPLC method for the determination of bisphenol A in water and serum samples. Multi-electrode electrochemical detection (ED) is widely used to analyze phenol compounds and neurotransmitters in water and biological samples. In general to analyze phenol compounds, the sensitivity and specificity of ED is far higher than that of FL and UV methods [11–13]. Therefore, we compared the methods of HPLC–UV and –FL and HPLC–ED to find the most sensitive method for the determination of bisphenol A.

The standard pretreatment for environmental bisphenol A has been done by liquid–liquid extraction with dichloromethane [6]. However, this method is not easy to operate and requires a large amount of an organic solvent. In this study the extraction was done by solid-phase extraction (SPE), because the SPE method is very easy and uses a small amount of an organic solvent. Silica-based reversed-phase sorbents, such as C_{18} or C_8, are the most widely used packings for SPE. However, they have various main limitations. If the sorbent runs dry anytime before the sample-loading step into the cartridges, the consequence is low and variable recovery [14–17]. Recently, Cheng et al. have demonstrated the SPE clean-up of a wide range of compounds using a hydrophilic–lipophilic balanced sorbent. With one generic SPE method, good recovery and excellent reproducibility are obtained for a wide range of compounds including acids, neutrals, bases, parent compounds and polar metabolites. Additionally, this SPE sorbent is fully wettable with water. Therefore, there is no impact of sorbent drying [18–20]. In this paper, the extraction for bisphenol A in water was done by two different sorbents, OASIS-HLB and Bond Elut Certify, and then are compared for SPE followed by bisphenol A in water. Bond Elut Certify (C_8/strong anion exchangers) has been chosen because they were used for the extraction of anionic species in tap water and river water [21]. In extraction for bisphenol A in human serum, the OASIS-HLB was done.

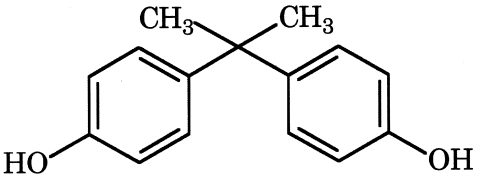

Fig. 1. Structure of bisphenol A: M_r 228.29.

In this study, we propose an easy and sensitive method for the determination of a trace amount of bisphenol A in water and human serum samples using HPLC–ED and SPE.

2. Experimental

2.1. Materials

Bisphenol A standard and pesticide analytical

grade of methanol, acetonitrile, acetone and dichloromethane were purchased from Wako (Japan). All solvents used for extraction and dilution were of pesticide analytical grade, and the others were of reagent grade. Water purified with a Milli-Q water purification system (Millipore, USA) was used. All experimental vessels were washed with acetone, followed by heating at 200°C for 4 h. The human serum samples were stored in blood bags.

A stock solution of 1.0 mg/ml of bisphenol A was prepared in acetonitrile, and prepared at 0.01~100 ng/ml as required by addition of acetonitrile–water (50:50).

2.2. HPLC–UV and –FL conditions

The HPLC system of a pump (LC 10AD) and a column oven (CTO-2A) at 40°C (Shimadzu, Japan) was used. The UV and FL detectors were SPD-6AV and RF-10 Axl from Shimadzu, respectively. Separation of the compounds was achieved by using a CAPCELL PAK UG 120 C_{18} (150×4.6 mm) column (Shiseido, Japan). Isocratic water–acetonitrile (60:40) was used as the mobile phase. The flow-rate was 1.0 ml/min. The injection volume was 50 μl. The detection wavelength was 217 nm for UV and excitation and emission wavelengths were 275 and 300 nm, respectively, for FL [5,7].

2.3. HPLC–ED conditions

The HPLC system consisted of a pump, an auto injector, and a column oven, LC 10AD (Shimadzu), Autosampler 460 (Kontron, USA) and CTO-2A (Shimadzu), respectively. The detection system consisted of a Coul Array MODEL 6210 multi-electrode electrochemical detector containing four cells (ESA, USA) and the database was Coul Array System ver. 1.0. Separation of the compounds was achieved by using a CAPCELL PAK UG 120 C_{18} (150×4.6 mm) column (Shiseido). The column oven was controlled at 40°C. The mobile phase was phosphoric acid–acetonitrile (60:40) and the flow-rate was 1.0 ml/min. The ED cell potentials were Ch_1 (350 mV), Ch_2 (430 mV), Ch_3 (570 mV) and Ch_4 (650 mV). The samples were injected in 50-μl amounts by use of an autosampler. After injection of each sample, acetonitrile was injected for monitoring a blank.

Peak confirmation was achieved by comparing the matching retention time and the peak ratio (R) between each standard and sample (R was the peak ratio of the dominant channel h_1/subdominant channel h_2) [11,12] (Fig. 2).

2.4. Investigation of bisphenol A contamination in experiment water

Water used for the experiments may be contaminated with bisphenol A because of plastics used in the water purifying equipment. It is thus necessary to investigate bisphenol A in water used for experiments to prepare bisphenol A-free water.

2.4.1. Preparation of bisphenol A-free water using an Empore disk

Water purified with a Milli-Q water-purification system was allowed to pass through an Empore disk to absorb bisphenol A to thereby prepare bisphenol A-free water. In order to examine the trapping ability of the Empore SDB-RPS disk (diameter 47 mm/0.5 mm) (3M, Japan), 1.0 ng/ml of bisphenol A was added to bisphenol A-free water, and some volume of the solution was allowed to pass through the disk. The thus-prepared bisphenol A-free water is referred to as the examination water hereafter.

2.4.2. Measurement of bisphenol A in experiment water

Bisphenol A in the passed solution was concentrated by liquid–liquid extraction using only glass labware. A 1-ml volume of 1 M HCl and 4.5 g of

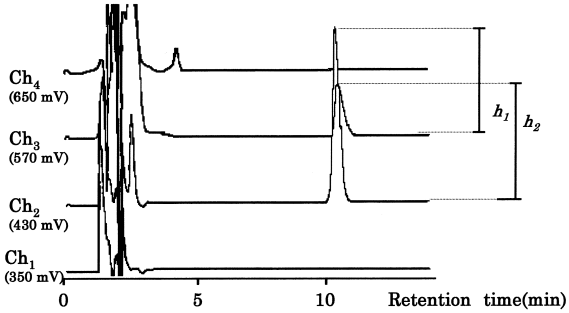

Fig. 2. Chromatogram from analysis of bisphenol A standard (100 ng/ml). The dominant channel (Ch_3) h_1/subdominant channel (Ch_2) h_2 is R.

sodium chloride were added to 600 ml of the water sample (examination water, tap water and Milli-Q water). A 30-ml volume of dichloromethane was added to the mixture, followed by vigorous shaking in a separatory funnel. The organic phase was collected in a 100-ml round bottomed flask. The aqueous phase was re-extracted with 30 ml of dichloromethane in the same way. The organic phase was combined with the first extract in a 100-ml round-bottomed flask, and then was evaporated to dryness with a rotary evaporator. The samples were reconstituted in 5 ml of acetonitrile–water (50:50). The obtained samples were measured by HPLC–ED.

2.5. Examination of pretreatment by solid-phase extraction

The standard pretreatment for environmental bisphenol A has been done by liquid–liquid extraction with dichloromethane [6]. However, this method is not easy to conduct and requires a large amount of organic solvent.

Accordingly, in this study the extraction was done by SPE, which is very easy to conduct and uses a small amount of organic solvent. However, it is not clear what materials the SPE cartridges are made of. Therefore, we tried to investigate bisphenol A contamination in SPE cartridges.

2.5.1. Investigation of bisphenol A contamination in SPE

We investigated bisphenol A contamination in the cartridges, OASIS HLB (130 mg/2.8 ml) (Waters, USA) and Bond Elut Certify (60 mg/3 ml) (Varian, USA), which are used in this pretreatment. The migration test was conducted by bathing cartridges of the filtration column (A), sorbent bed (B) and frits (C) in 100 ml of methanol for 30 min at room temperature. Then, 10 ml of methanol was evaporated to dryness with a rotary evaporator. The samples were reconstituted in 100 μl of acetonitrile–water (50:50).

2.5.2. Extraction of bisphenol A removal in the operating process

We measured bisphenol A in the cartridges used for extraction. A 3-ml volume of methanol was allowed to pass through the cartridges, and then concentrated, followed by reconstitution in 100 μl of acetonitrile–water (50:50).

2.5.3. Extraction of bisphenol A in water and serum samples using SPE

The water and serum samples were extracted using SPE cartridges. The extraction cartridges were conditioned with 15 ml of methanol followed by 3 ml of examination water. After 1 ml of 1 *M* HCl, 1 ml methanol, and 5 ml of examination water were added to 3 ml of the serum samples, the samples were vortex-mixed for 10 min and then applied onto the conditioned SPE cartridges under a vacuum. The cartridges were washed with 3 ml of water–methanol (90:10), and then eluted with 3 ml of methanol. The SPE elution was evaporated to dryness and the dried residues were reconstituted in 100 μl of acetonitrile–water (50:50). For the recovery test, standard solutions were added to the water samples so that they contained bisphenol A in an amount of 0.5 or 0.05 ng/ml, and to the serum samples so that they contained bisphenol A in an amount of 1.0 or 0.1 ng/ml.

3. Results and discussion

3.1. Optimization of HPLC–ED

ED was adopted because of the well known electroactivity of the phenolic group present in bisphenol A molecule. It has been stated that phenolic oxidation potentials generally shift to more negative values with an increasing pH. The pH of the supporting electrolyte may also influence the electron-transfer rate constants. For that reason, we chose phosphoric acid for the mobile phase. Acetonitrile–phosphoric acid in water was used as the mobile phase and the sensitivity of detection was adjusted to good sensitivity depending on the concentrations of phosphoric acid. Hydrodynamic voltammograms of bisphenol A under varying concentrations of phosphoric acid conditions are shown in Fig. 3. Based on the results, we decided to use 0.3% phosphoric acid in water.

Under these conditions, the detection limit was 0.01 ng/ml (0.5 pg). The calibration curve for bisphenol A standard constructed by plotting the

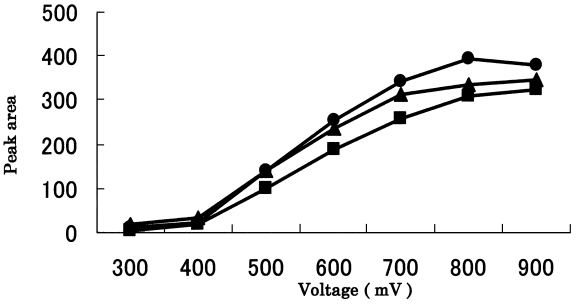

Fig. 3. Hydrodynamic voltammograms of bisphenol A (500 ng/ml). Apply voltage; Ch_1 (100 mV) and Ch_2 (detector voltage) ▲: 0.4% H_3PO_4–CH_3CN (60:40), ●: 0.3% H_3PO_4–CH_3CN (60:40), ■: 0.2% H_3PO_4–CH_3CN (60:40).

concentration versus the peak area showed a good linearity in the 0.1~100 ng/ml range (R=0.999). When the bisphenol A standard was consecutively measured for 5.0, 50 ng/ml, nine times, the retention times were 10.4 min (RSD=0.05%) with the peak ratio RSD of 4.86% and 10.4 min (RSD=0.07%) with that of 0.77%, respectively.

Gonzalez-Casado and co-workers stated that their method to calculate the detection limit for the determination of chemicals was more in line with the IUPAC recommendations [10,22]. Likewise, our method to calculate the detection limit for the determination of bisphenol A was according to the IUPAC recommendations. Our method is assumed to have the highest sensitivity among HPLC methods requiring no derivatization. To confirm this, we compared our method with the typical detection methods for bisphenol A, UV and FL detection.

3.2. Comparison of UV and FL detection

A study, to compare the detection of bisphenol A with UV, FL and ED, was carried out. The detection limits of UV and FL detection were 30.0 ng/ml (1.5 ng) and 2.0 ng/ml (100 pg). When the bisphenol A standard was consecutively measured for 50 ng/ml, nine times, it was shown that the retention times were 10.5 min (RSD=1.07%), and 10.6 min (RSD=0.84%), respectively.

Table 1 indicated different analytical parameters of UV, FL and ED of bisphenol A. The detection limits for UV, FL and ED methods showed that the sensitivity ratios of UV/ED and FL/ED, were more than 3000- and 200-times larger than the detection limits of UV and FL methods.

3.3. Investigation of bisphenol A contamination in the experiment water

We encountered difficulties that bisphenol A was detected when SPE was used as a blank, suggesting that SPE was contaminated by bisphenol A. For this reason, we would like to investigate the contamination of bisphenol A in experimental processes and water. It was necessary to prepare a bisphenol A-free water for the pretreatment. Thus, bisphenol A-free water was prepared using an Empore disk. The trapping ability of bisphenol A was examined by use of an Empore disk. When the trapping ability of an Empore disk against 1.0 ng/ml of bisphenol A solution was investigated, 100% of 2 l or less of bisphenol A solution water was trapped. Based on this result, water obtained by allowing Milli-Q-purified water to pass up to 2 l was used as bisphenol A-free water for the determination of a trace amount of bisphenol A. The thus-obtained water was referred to as examination water thereafter.

Then, bisphenol A in tap water, Milli-Q-purified water, and examination water was determined by liquid–liquid extraction. The concentrations of bisphenol A in tap water and Milli-Q-purified water

Table 1
Analytical parameters of HPLC

Parameter	UV	FL	ED
Detection limit (pg)	1500	100	0.5
RSD of retention time (%)	1.07	0.84	0.07
Quantitation limit of water sample (ng/ml)	30.0	2.0	0.01
Quantitation limit of serum sample (ng/ml)	150.0	10.0	0.05
Linear range (ng/ml)	50–500	10.0–200	0.1–100
Correlation coefficient (R)	0.989	0.987	0.999

were 0.01 and 0.02 ng/ml, respectively. Bisphenol A was not detected in the examination water, obtained by using an Empore disk. When Milli-Q-purified water, which is normally used in experiments, is used in the pretreatment for the determination of a trace amount of bisphenol A, it may cause contamination. Bisphenol A-free water should be used instead. Thus, we developed a sensitive method for the determination of a trace amount of bisphenol A in water sample using the examination water.

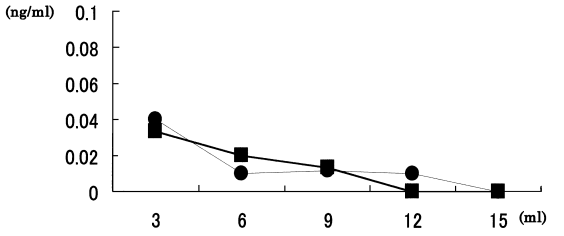

Fig. 4. Remaining bisphenol A in SPE cartridges eluted with methanol ●: OASIS HLB, ■: Bond Elut Certify.

3.4. Pretreatment of bisphenol A from SPE

3.4.1. Investigate of bisphenol A contamination in SPE cartridge

In this study, the extraction was done by SPE. However, using SPE cartridges for the pretreatment was a cause of contamination by bisphenol A. Thus, bisphenol A in two types of SPE cartridges, Oasis HLB and Bond Elut Certify, was measured by HPLC–ED, as shown in Table 2. A trace amount of bisphenol A was detected. The detected bisphenol A was probably derived not from the material but from the contamination in the preparation process. In order to remove bisphenol A, we tried prewashing the cartridges with a large amount of an organic solvent for conditioning in the SPE process. Fig. 4 shows the concentrations of bisphenol A contamination in the SPE eluted with methanol. It was found that bisphenol A was perfectly removed when prewashed with at least 15 ml of methanol.

3.4.2. Pretreatment of bisphenol A in water and serum samples using the SPE method

Therefore, the extraction cartridges were conditioned with 15 ml of methanol followed by 3 ml of water. Then, samples were applied onto the conditioned SPE system. The extraction cartridges were washed with water–methanol (90:10), and then eluted with methanol. Table 3 shows the recovery test of 0.05, 0.5 ng/ml of bisphenol A in water using OASIS HLB and Bond Elut Certify. Recoveries of bisphenol A by both types of cartridges, were greater than 80%, but the results using OASIS HLB were 98.2% and 95.5% and better than the results using Bond Elut Certify.

The limit of quantitation of our method for the determination of bisphenol A in water sample was 0.01 ng/ml, which satisfies the limit of quantitation of The European Union.

3.5. Determination of bisphenol A in human serum

The method was applied to the determination of bisphenol A at low concentration levels in human serum sample. The recovery tests of 0.1 and 1.0

Table 3
Recovery test of 0.5, 0.05 ng/ml of bisphenol A in examination water[a]

ng/ml	Recovery (%) [RSD, %]	
	OASIS HLB	Bond Elut Certify
0.05	98.2 [3.4]	92.3 [5.3]
0.5	95.5[6.1]	83.8 [3.5]
Control	ND	ND

[a] ND<0.01 ng/ml, $n=6$.

Table 2
Migration concentration of bisphenol A from SPE cartridges[a]

	Bisphenol A concentration (ng/ml) [RSD, %]		
	A	B	C
OASIS HLB (60 mg/3 ml)	0.013 [33.8]	ND	0.011 [20.0]
Bond Elut Certify (130 mg/2.8 ml)	0.013 [27.5]	ND	0.010 [42.4]

[a] ND<0.01 ng/ml, $n=6$.

Table 4
Recovery tests of 1.0, 0.1 ng/ml of bisphenol A in serum samples by OASIS HLB[a]

ng/ml	Recovery (%) [RSD, %]
0.1	87.3 [13.5]
1.0	79.0 [5.1]
Control	0.16 ng/ml

[a] ND<0.05 ng/ml, $n=6$.

ng/ml in the control serum using OASIS HLB showed 79.0 and 87.3%, respectively (Table 4). Based on our analysis of bisphenol A in the human serum, the concentration was 0.32 ng/ml ($n=5$, RSD 5.0%). Sajiki et al. reported that bisphenol A concentrations in healthy human serum were low (0–1.6 ng/ml) [23]. Consequently, it is reasonable to suppose that free bisphenol A concentration in healthy human serum is low. It may be explained by metabolism of bisphenol A in human serum. Bisphenol A has been reported to be hydroxylated in vivo [24] and be glucuronidated in the male rat liver [25].

In conclusion, this method for the determination of bisphenol A using HPLC–ED with SPE has a high sensitivity and selectivity, and can be applied to bisphenol A in the serum and to other biomaterials.

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