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Measurement of bisphenol A levels in human blood serum and ascitic fluid by HPLC using a fluorescent labeling reagent

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

A sensitive column-switching HPLC method with fluorescence detection was developed for the determination of bisphenol A (BPA) in human blood serum and ascitic fluid samples. 4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) was used as the fluorescent label, and the excess reagent was removed by a column-switching technique. Liquid–liquid extraction with chloroform was used for the pretreatment of serum and ascitic fluid samples. BPA in both the samples could be determined in the concentration range of 0.1–7.0 ppb with the detection limit of 0.04 ppb at a signal-to-noise ratio of 3. The recoveries of BPA spiked to serum and ascitic fluid were 78.6 and 77.7%, respectively. The mean concentrations of BPA ($n = 9$) in maternal and umbilical cord blood sera obtained from healthy pregnant women were 0.46 ± 0.20 and 0.62 ± 0.13 ppb, respectively. BPA levels ($n = 21$) in blood sera and ascitic fluid obtained from the patients with sterility were also determined to be 0.46 ± 0.20 and 0.56 ± 0.19 ppb, respectively. Relationships of BPA concentrations were observed between maternal and umbilical cord blood serum samples ($r = 0.626$), as well as blood serum and ascitic fluid samples ($r = 0.785$).

Keywords: Bisphenol A; Column-switching HPLC; Fluorescence detection; Cord blood serum; Ascitic fluid

1. Introduction

Bisphenol A (BPA 4,4′-Isopropylidenediphenol) has been used in the chemical industry for the production of polycarbonate plastics, epoxy resins

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and other resins. These are extensively employed for polycarbonate bottles and containers, food can linings, and white dental fillings and sealants. Human exposure to BPA may arise through BPA leaching from these materials into foods [1,2] and saliva [3,4]. Since a weak estrogen-like activity of BPA was reported by Krishnan et al. in 1993 [5], its effects on human health have become of growing concern. BPA orally administered could easily cross the placental barrier and enter the fetus in animal experiment [6]. It was reported that fetus exposure to BPA in the womb caused an increase in prostate weights [7] and a decrease in daily sperm production [8] for male mice, and earlier puberty for female mice [9]. Effects of BPA on rat brain development in offspring were also reported [10]. BPA gives rise to adverse biological effects even at low levels especially in fetus because the sex hormones play a crucial role in its development. Therefore, a sensitive and selective method for the determination of BPA in maternal and umbilical cord blood serum is of great importance to assess placental transport of BPA and its prenatal exposure.

In the previous papers, we reported a highly fluorescent labeling reagent for phenols, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl), that was used to HPLC analyses of phenol and chlorophenols in human urine [11], and BPA migrated from polycarbonate baby bottles [12]. Recently, we developed a column-switching HPLC method in order to remove the excess reagent and it was successfully applied to the sensitive monitoring of BPA concentrations in rat brain microdialysate obtained after oral and i.v. administration of BPA [13].

In the present study, the column-switching HPLC method was modified to be applicable to blood serum and ascitic fluid samples. BPA concentrations in maternal and umbilical cord blood sera for healthy pregnant women were measured by the proposed method, and the relation of the BPA levels in both the blood samples was investigated. Moreover, we revealed the BPA levels in ascitic fluid and blood serum samples obtained from the patients with sterility.

2. Experimental

2.1. Reagents and chemicals

BPA, chloroform, triethylamine, 25% ammonia solution, hydrochloric acid and acetic acid of reagent grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Acetonitrile and methanol were of HPLC grade (Wako). DIB-Cl was synthesized in our laboratory as reported previously [14], which is now available from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Water was deionized, distilled and passed through a water purification system (Puric-Z, Organo Co., Tokyo).

2.2. Subjects

Nine sets of maternal and umbilical cord blood serum were collected from healthy pregnant women. Samples of cord blood were obtained at the time of delivery. Twenty-one sets of peripheral blood serum and ascitic fluid were withdrawn from the patients with sterility. Glass syringes and glass tubes were employed throughout the sampling to avoid a contamination of BPA. Serum and ascitic fluid samples were stored at −20 °C in glass tubes until use. Adequate informed consents were given to all donors of blood and ascitic fluid samples for the monitoring of BPA levels.

2.3. HPLC system

The HPLC system (Fig. 1) consisted of two pumps (LC-10AS, Shimadzu, Kyoto, Japan), a Shimadzu RF-10A_{XL} fluorescence detector, a 7125 injector with a 5-μl loop (Rheodyne, Cotati, CA), a SPV-N-6 column-switching valve with a 200-μl loop (GL Sciences, Tokyo, Japan), and a Tosoh FBR-1 recorder (Tokyo, Japan). The mobile phases used were mobile phase 1: acetonitrile–H_2O–methanol (72:13:15, v/v) for pump 1 and mobile phase 2: acetonitrile–0.1 M acetate buffer (pH 5.5)–methanol (55:12:33, v/v) for pump 2, which were delivered at flow rates of 0.10 and 0.30 ml/min, respectively. The temperature for both columns was maintained at 35 °C in a column oven (Omron E5C3, Tokyo). Sample was injected

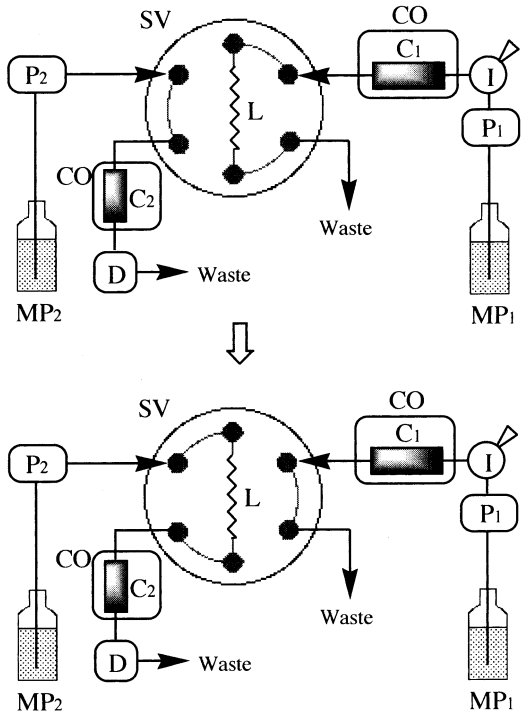

Fig. 1. Schematic diagram of the column-switching HPLC system. Pumps, P1 and P2; injector, I; switching valve, SV; loop, L; columns, C1 (precolumn) and C2 (separation column); column oven, CO; detector, D; mobile phase, MP1 (mobile phase 1) and MP2 (mobile phase 2).

onto column 1 (precolumn, Wakosil-II 5C18, 150 × 1.0 mm i.d., Wako) and purged with mobile phase 1 to pass through the loop of column-switching valve. After an injection time of 10.75 min, the valve was switched and then the analyte was loaded onto column 2 (separation column, Protein & Peptide C18, 150 × 4.6 mm i.d., Vydac, Hesperia, CA) and eluted with mobile phase 2 to the fluorescence detector. This valve position was kept till an analysis was finished. Fluorescence intensity was monitored at 475 nm with an excitation of 350 nm.

2.4. Assay procedure

A 100-μl portion of serum or ascitic fluid sample was transferred into a glass stoppered centrifuge tube, and to this were added 100 μl of 0.2 M HCl and 1.0 ml of chloroform. The mixture was vortex-mixed for 1 min and then centrifuged at 1000 × *g* for 25 min at 4 °C. The organic layer (0.85 ml) was collected and evaporated to dryness with a centrifugal evaporator (model RD-31,Yamato Kagaku, Tokyo). To the residue, 100 μl of 5 mM DIB-Cl suspension in acetonitrile and 5 μl of 1.5 M triethylamine were added, and reacted for 20 min at 35 °C. Then, 10 μl of 12.5% of ammonia solution was added to the reaction mixture to stop the reaction. After 10 min, 10 μl of 5% acetic acid was added for neutralization. A 5-μl portion of the resultant reaction mixture after passing through a membrane filter (0.45 μm, HLC-DISK 3, Kanto Chemical Co. Inc., Tokyo) was injected onto the HPLC system. Fig. 2 shows the labeling reaction of BPA with DIB-Cl.

3. Results and discussion

3.1. Chromatographic conditions

In the previous paper, we developed a column-switching HPLC method for the determination of BPA in rat brain microdialysate and rat plasma obtained after oral and i.v. administration of BPA, in which column-switching technique was introduced as an on-line clean up procedure for removing excess fluorescent labeling reagent [13]. The method enabled highly sensitive detection of BPA (detection limit of 0.3 ppb at a signal-to-noise ratio of 3 in 60 μl rat brain microdialysate) for a microdialysate sample that includes relatively simple compositions. However, the sensitivity of the method (detection limit of 4.6 ppb in 50 μl rat plasma) was not enough to be applied to small amount of human serum samples. Therefore, we modified this method to be more sensitive to allow the monitoring of ordinary levels of BPA in human blood serum and ascitic fluid. In the present study, a loop of larger size (200 μl) attached to the switching valve and a separation column of regular size (150 × 4.6 mm i.d.) were employed instead of the previous loop (20 μl) and a semimicro-separation column (Develosil ODS-5, 250 × 1.5 mm i.d., Nomura Chemical Co. Ltd, Seto, Japan), respectively. By these modifications, a larger sample zone obtained by separation with

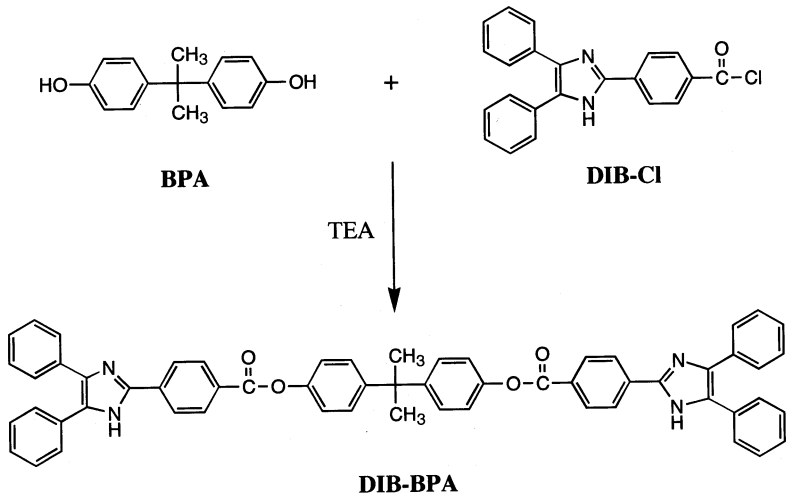

Fig. 2. Reaction scheme for labeling BPA with DIB-Cl.

the precolumn could be effectively introduced into the separation column, which resulted in the increase in the sensitivity for BPA detection.

The effect of the column-switching time was studied over a range of 10.00–11.00 min. The maximum and constant peak height was observed at the range of 10.00–10.75 min, and then slightly decreased with expansion of the switching time; 10.75 min was selected as optimum. BPA was eluted at 11.60 min after the valve switching and the peak for BPA could be separated from interference peak under this condition. Fig. 3 shows the representative chromatograms obtained from reagent blank, cord blood serum, ascitic fluid and ascitic fluid spiked with 3.0 ppb of BPA.

3.2. Validation of the method

Table 1 summarizes the calibration curves parameters and detection limits. For both the serum and ascitic fluid samples, the linear relationships between the peak heights and spiked concentrations were obtained over a range of 0.1–7.0 ppb ($n = 7$). Lower detection limits (LOD) of BPA were 0.04 ppb for both samples at a signal-to-noise ratio of 3. This sensitivity was almost 100 times higher than that of the previous method with regard to blood sample [13]. When compared to other analytical methods for BPA in biological samples, the sensitivity of the proposed method is slightly higher than those of a GC-MS method for human urine (LOD: 0.12 ppb) [15], an HPLC method with electrochemical detector (LOD: 0.2 ppb) [16,17] and an HPLC-MS method (LOD: 0.1 ppb) for human serum [16], and almost comparable to that of an HPLC method with multi-electrode electrochemical detection for human serum (LOD: 0.05 ppb) [18].

Recoveries were calculated by comparing the peak heights of the spiked analytes (3.0 ppb) with that of the standard solution at the same concentration of BPA in which the standard solution was subjected to the procedure for the sample pretreatment including an extraction step. Both the samples showed almost the same recovery: 78.6% for serum and 77.7% for ascitic fluid.

Precision of the proposed method was examined on serum samples spiked with 1.0 ppb of BPA. The results of precision study was satisfactory in which the relative standard deviations (RSDs) for within-($n = 6$) and between-day ($n = 3$) analyses were 4.2 and 8.0%, respectively.

3.3. Determination of BPA in serum and ascitic fluid

The proposed method was applied to the monitoring of BPA levels in human maternal and

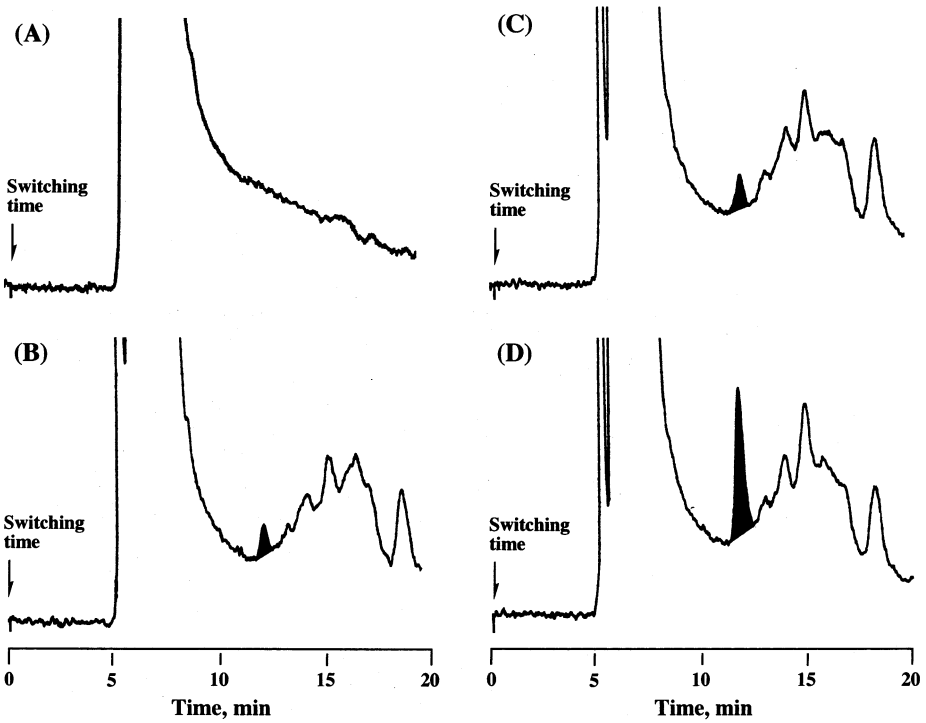

Fig. 3. Typical chromatograms corresponding to (A) reagent blank, (B) blood serum (BPA concentration: 0.81 ppb), (C) ascitic fluid (BPA concentration: 0.88 ppb) and (D) the ascitic fluid spiked with 3.0 ppb of BPA.

umbilical cord blood sera that were taken from nine healthy pregnant women. The results are shown in Table 2. The levels of BPA were in the range from 0.21 to 0.79 ppb with the mean concentration of 0.46 ± 0.20 ppb for maternal serum and in the range from 0.45 to 0.76 ppb with the mean concentration of 0.62 ± 0.13 ppb for cord blood serum. BPA level in maternal serum of 0.46 ± 0.20 ppb is in good agreement with reported values on human serum samples, i.e. 0.33 ± 0.54 ppb for women and 0.59 ± 0.21 ppb for men [16], and 0.32 ppb for healthy human serum [18]. The mean concentration of BPA in cord blood serum was significantly higher than that in maternal serum ($P < 0.02$). BPA levels in both the samples were tested for significance by paired Student's t-tests. It was reported that lipid and lipoprotein profiles were different between cord blood and adult blood [19]. This might contribute to the difference in BPA levels in both the blood samples. However, more experimental and clinical sample measurements will be required to elucidate the unknown contributions. As shown in Fig. 4, a weak positive correlation ($r = 0.626$) between ma-

Table 1
Calibration curves parameters and detection limits for serum and ascitic fluid samples

Sample	Concentration (ppb)	Regression equation[a]	Correlation coefficient	Detection limit (ppb)[b]
Serum	0.1–7.0	$y = 1.27x - 0.08$	0.998	0.04
Ascitic fluid	0.1–7.0	$y = 1.22x + 0.29$	0.999	0.04

[a] y as peak height (cm) and x as concentration in ppb.
[b] Signal-to-noise ratio of 3.

Table 2
Concentrations of BPA in maternal and umbilical cord blood sera

Sample no.	BPA concentration in serum (ppb)	
	Maternal blood	Cord blood
1	0.43	0.61
2	0.23	0.45
3	0.63	0.69
4	0.65	0.76
5	0.45	0.45
6	0.43	0.74
7	0.34	0.49
8	0.79	0.73
9	0.21	0.64
Mean ± S.D.	0.46 ± 0.20	0.62 ± 0.13
Median	0.43	0.64
Range	0.21–0.79	0.45–0.76

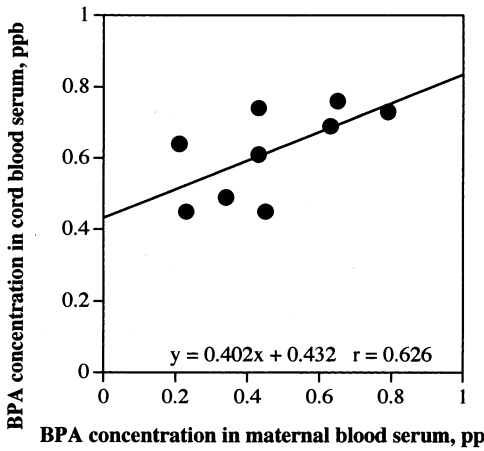

Fig. 4. Correlation between the concentrations of BPA ($n = 9$) in maternal and cord blood sera.

ternal and cord blood serum levels of BPA was observed.

BPA levels in peripheral blood serum and ascitic fluid obtained from the patients with sterility were also determined by the method (Table 3). The mean level of BPA in ascitic fluid (0.56 ppb ± 0.19) is a little but significantly ($P < 0.002$) higher than that in peripheral blood (0.46 ± 0.20 ppb). However, there was no difference in the mean concentrations of BPA between blood sera from the patients with sterility and healthy pregnant women

Table 3
Concentrations of BPA in blood sera and ascitic fluid

Sample no.	BPA concentration (ppb)	
	Blood serum	Ascitic fluid
1	0.30	0.75
2	0.81	0.88
3	0.23	0.35
4	0.87	0.78
5	0.36	0.55
6	0.46	0.63
7	0.49	0.61
8	0.31	0.62
9	0.44	0.50
10	0.22	0.22
11	0.24	0.15
12	0.85	0.85
13	0.24	0.43
14	0.47	0.73
15	0.64	0.64
16	0.46	0.56
17	0.40	0.49
18	0.38	0.42
19	0.51	0.56
20	0.56	0.71
21	0.33	0.38
Mean ± S.D.	0.46 ± 0.20	0.56 ± 0.19
Median	0.44	0.56
Range	0.22–0.87	0.15–0.88

(0.46 ppb, Table 2). The contribution of BPA to sterility could not be confirmed by these results. A relatively strong correlation ($r = 0.785$) was ob-

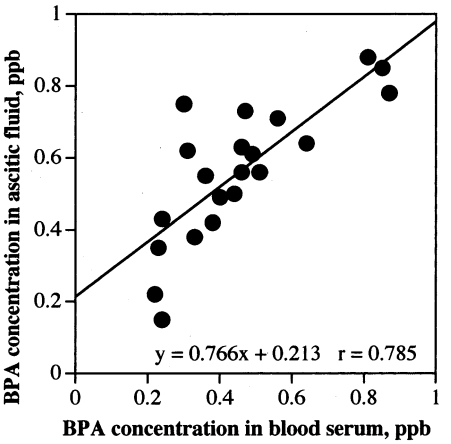

Fig. 5. Correlation between the concentration s of BPA ($n = 21$) in peripheral blood sera and ascitic fluid.

served between BPA concentrations in peripheral blood sera and ascitic fluid (Fig. 5): this may suggest some association between the distributions of BPA in both the biological fluids.

In conclusion, a column-switching HPLC method with fluorescence detection was developed for the determination of BPA in human blood serum and ascitic fluid samples. The method is highly sensitive in which as low as 0.04 ppb of BPA in serum and ascitic fluid samples can be detected. The determination of BPA in maternal and umbilical cord blood sera from healthy pregnant women, as well as in ascitic fluid and blood sera from the patients with sterility could satisfactorily be performed by the proposed method. The method should be useful to estimate the fetus and adult exposure to BPA.

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