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Journal of Chromatography B, 755 (2001) 9–15

JOURNAL OF CHROMATOGRAPHY B

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Determination of bisphenol A in blood using high-performance liquid chromatography-electrochemical detection with solid-phase extraction

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Received 23 May 2000; received in revised form 29 August 2000; accepted 1 September 2000

Abstract

A method for the determination of bisphenol A (BPA) in blood was investigated using high-performance liquid chromatography-electrochemical detection (HPLC-ED) with solid-phase extraction. When BPA at the concentrations of 25–100 ng/ml were added to whole blood, BPA recoveries were 26–48%. When BPA was added to water, plasma or hemolyzed red blood cells (H-RBC), BPA recoveries in water and plasma were almost similar (94%). However, the recovery in H-RBC was very low (36–46%). When BPA and plasma were added to H-RBC, the recovery was 70–85%. In authentic bovine metHb solution, BPA decreased depending on the metHb concentration, however, BPA recovery in the solution added with more than 17% plasma was higher than that in metHb only. These suggest that metHb influences the BPA recovery in whole blood. However, an accurate determination of BPA using HPLC was easily made possible by separating RBC from plasma.

Keywords: Bisphenol A; Methemoglobin

1. Introduction

Bisphenol A (BPA), a compound widely used as the monomer for the production of polycarbonate plastics such as baby bottles, and as a major component of epoxy resin for the lining of food cans and dental sealants, is well known as a strong estrogenic endocrine-disrupting agent [1,2].

Recently it was reported that exposure of pregnant mice to even low levels BPA (2 or 20 ng/g body weight) resulted in not only a reduction of daily sperm production, epididymal weight and an enlargement of prostate of male offspring [3,4], but also in an advanced puberty such as increase of body weight at weaning and shortness of time of first oestrus in female offspring [5]. Since BPA is easily leached in biological samples such as serum and saliva from polycarbonate plastic bottles [6] and resin-based dental composites [7], it is likely to have an adverse effect on humans. Therefore, determining BPA accurately in biological samples is necessary.

Generally, natural steroidal estrogens such as 17β-estradiol circulate in the body associated with the serum-binding proteins, sex hormone-binding globulin and albumin, and only a small fraction is free. On the other hand, BPA is considered to exist in serum as a free type like other xenoestrogens [8–10].

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00489-8

Our study [6] using an HPLC method with solid-phase extraction showed that the recovery of BPA in serum was high (above 93%), suggesting that BPA exists as free type in serum. However, few studies on the determination of BPA in whole blood were reported.

An HPLC system with an electrochemical detector (ED) is now widely used to analyze phenol compounds in biological samples [11,12], and it has been confirmed that this system has some advantages, e.g., high sensitivity, good reproducibility and easiness of handling for the determination of BPA in serum [6]. Since BPA is a phenol compound, and was successfully determined in serum by HPLC-ED, this method is thought to be appropriate for BPA analysis in whole blood in this study. Therefore, HPLC-ED with solid-phase extraction was used throughout the experiments. Furthermore, the influence of hemolysis in serum on the BPA value were investigated.

2. Experimental

2.1. Materials

Fresh sheep blood was collected from the leg vein of three sheep, which were raised domestically, and was preserved at 5°C. All experiments were done within 2 weeks after blood collection. Human defibrinized blood and sera were collected from healthy volunteers. BPA was purchased from Wako Pure Chemical (Tokyo, Japan). Crystallized and lyophilized bovine methemoglobin (metHb) was purchased from Sigma (St. Louis, MO, USA). Throughout the experiment, BPA-free water which was prepared using ODS-silica Sep-pak (Waters, MA, USA) was used. An Oasis HLB (Waters) used for the solid-phase extraction of BPA was washed with 3.5 ml ethanol (EtOH) and 3.5 ml water before use.

2.2. Preparation of samples

2.2.1. BPA concentrations in a BPA added sheep whole blood

One-tenth ml BPA (0.275–1.1 μg/ml) dissolved in saline was added to 1 ml of defibrinized blood or water. For the control, saline was added to either water or blood instead of BPA. One-fifth ml of the blood samples was diluted with water to a final volume of 5 ml at 10 min after addition of BPA.

2.2.2. BPA concentrations in a BPA sheep plasma and H-RBC

Plasma was separated from RBC by centrifuging at 1000 *g* for 10 min immediately after the collection. RBC was washed 4 times with 10 volumes of saline and hemolysis was done by adding 4-fold water (H-RBC). Two sets of experiments were carried out: (A) H-RBC was added with plasma; (B) H-RBC was added with water. Two ml plasma or water were added to each 4 ml H-RBC. Each set was divided into two groups, and to each group was added either 66.6 ng/ml BPA (final concentration) or the same volume of water. Experiments were carried out at 37°C for 24 h. A total of 0.2–0.5 ml of plasma or H-RBC was diluted with water to a final volume of 5 ml.

2.2.3. Effect of plasma on BPA recovery in bovine metHb solution

Authentic bovine metHb was dissolved in water at a concentration of 5×10^{-3}–5 mg/ml. A 0.5 ml metHb solution was added to 0.1 ml sheep plasma and/or 25 ng/ml BPA (final concentration). To investigate the effect of plasma concentration on BPA recovery, plasma was added to 5 mg/ml metHb solution containing of 25 ng/ml BPA (final concentration) at a concentration of 17–93%. The samples were allowed to stand for 30 min at room temperature and were diluted with water to a final volume of 5 ml.

2.2.4. Effect of oxidative agents on BPA concentration in H-RBC

Fifty μl of sodium nitrite (0–2.5 mg/ml in water), the oxidative agent changing oxyHb to metHb, was added to either 0.2 ml sheep H-RBC or water containing 300 ng/ml BPA (final concentration). The samples were allowed to stand for 30 min at room temperature and were diluted with water to a final volume of 5 ml.

2.2.5. Effect of metHb on the determination of BPA in serum

MetHb at a concentration of 0.5–2.5 mg/ml was added to non-hemolytic human serum containing 40

ng/ml BPA. The samples were allowed to stand for 10 min at room temperature and were diluted with water to a final volume of 5 ml. Another experiment where human sera were grouped in three hemolytic degrees (none, medium with pinkish color and strong with reddish color) and each sera were pooled, was prepared. BPA at a concentration of 50 ng/ml was added to 0.5 ml of each of the three samples, and the samples were allowed to stand for 1 h at room temperature. Sample tubes were filled up to 5 ml with water.

2.3. Analytical parameters

2.3.1. Extraction and determination of BPA

Extraction of BPA was carried out according to the method of Sajiki et al. [6]. All samples were applied to Oasis, and polar lipids were removed from the column with 3.5 ml 15% EtOH and washed with 3.5 ml water. To remove nonpolar lipids, 3.5 ml petroleum ether was used. Finally, BPA was eluted with 3.5 ml ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in 1 ml acetonitrile–water (40:60). BPA standards were prepared with water using the 1 μg/ml stock solution which was dissolved with 25% methanol (MeOH).

HPLC, Model LC-10 AD (Shimadzu, Kyoto, Japan) with Shim-Pack VP-ODS column (150×4.6 mm I.D., Shimadzu), and electrochemical detector (ED, Coulochem II 5200A, ESA, MA, USA) were used for BSA analysis. The solvent system was acetonitrile–water–phosphoric acid (40:60:0.2). The flow-rate and column temperature were 1.0 ml/min and 40°C, respectively. The injection volume was 50 μl. Conditions of ED were guard cell potential, E=600 mV; analytical cell potentials, E_1=300 mV and E_2=550 mV; sensitivity, 1 μA, respectively. Identification of BPA was made by comparing the HPLC retention times with those of the authentic standards. Further, co-chromatography using authentic standards was employed for a complete identification.

2.3.2. Determination of oxyhemoglobin (oxyHb) and methemoglobin (metHb)

MetHb concentration in H-RBC was measured by taking the difference between optical densities at 630 nm (a peak of metHb) and at 700 nm (base line). OxyHb concentration in serum was measured by taking the difference between optical densities at 540 nm (a peak of oxytHb) and at 560 nm [13]. Bovine metHb was used as metHb standard.

2.3.3. Statistical analysis

Data were analyzed by one-way analysis of variance, and the difference among treatments was tested by least significant difference. Test of significance between two groups was done by Student's *t*-test.

3. Results

BPA concentrations in whole blood added with 0–100 ng/ml BPA are shown in Table 1. MetHb concentration of whole blood was 3.7 mg/ml. BPA recoveries in water with added BPA, 25, 50, 75 and 100 ng/ml, were 97, 94, 95 and 96%, respectively. The recoveries in whole blood added with the same concentration of BPA were 25, 27, 47 and 46%, respectively. Significant difference ($P<0.01$) between water and whole blood was observed in all added BPA concentrations. BPA recovery in the blood sample decreased depending on the logarithm value of added BPA concentration. Table 2 shows the change of BPA concentrations in blood fractions at 10 min and at 1 day after addition of BPA at 37°C. BPA was not detected in either plasma or H-RBC on the day they were collected from sheep. When 66.6 ng/ml BPA were added to each blood fraction and allowed to stand for 1 day at 37°C, the BPA concentration in plasma was 63.5 ng/ml, which was

Table 1
Change of BPA concentrations in water or whole blood added with various dose of BPA

Added BPA (ng/ml)	BPA concentration (ng/ml) [a]		
	Water	Whole blood	Significance[b]
0	0	0	
25	24.3	6.3	**
50	47.0	13.5	**
75	71.2	35.5	**
100	96	46.0	**

[a] Data represent the mean values of triplicates. Samples were allowed to stand for 10 min at room temperature.

[b] Significant difference between water and whole blood in the same line: ** $P<0.01$.

Table 2
BPA concentrations in sheep blood fractions at days 0 and 1 after addition of BPA[a]

	Non-BPA		Added BPA (66.6 ng/ml)	
	Day 0	Day 1	Day 0	Day 1
(1) water	0	0	63.5^{x}	63.2^{x}
(2) plasma	0	0	62.5^{x}	63.5^{x}
(3) H-RBC[b]				
− plasma	0	0	23.7^{y}	30.5^{y}
+ plasma	0	2.2	45.0^{z}	56.5^{x}

[a] Data represent the mean values (ng/ml) of duplicates. Samples were allowed to stand at 37°C.
[b] RBC diluted 5-fold with water.
Different superscript letters in the same column indicate a significant difference: $P<0.01$.

a little higher than that in water (63.2 ng/ml). The BPA concentrations in H-RBC at 10 min and 1 day after BPA addition decreased significantly ($P<0.01$) to 23.7 and 30.5 ng/ml, respectively. BPA concentrations in H-RBC simultaneously added with plasma at a concentration of 50% at both 10 min and 1 day after BPA addition were 45.0 and 56.5 ng/ml, respectively. Thus, addition of sheep plasma inhibited the decrease of BPA recovery in H-RBC. Addition of human serum instead of sheep plasma also brought the same inhibitory effect on the decrease of BPA. A total of 2.2 ng/ml BPA which corresponded to the concentration of 18.3 ng/ml in RBC, was detected in H-RBC added with plasma 1 day after BPA addition. To investigate whether the change in BPA concentration relates to the existence of Hb or not, an experiment using crystallized bovine metHb solution (stable type of Hb in aerobic condition) was carried out. When 4.2 mg/ml metHb was added to water containing 25 ng/ml BPA, BPA concentration decreased significantly ($P<0.01$) to 18% within 10 min after the addition, but it recovered to 53% in 20 min and maintained that level until 80 min (Fig. 1, bottom left). In Fig. 1, BPA concentration after 30 min of addition to metHb decreased as a function of metHb concentration (42–

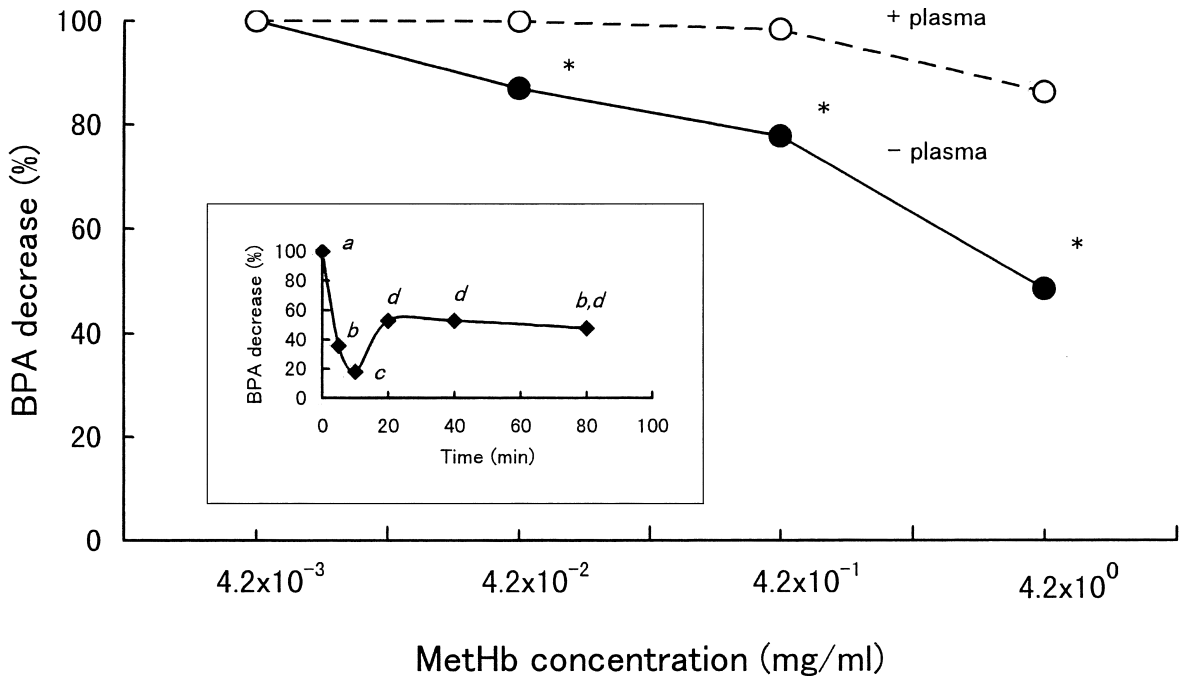

Fig. 1. Changes of BPA concentration as a function of metHb concentration in H-RBC with or without plasma. Data represent the mean values of triplicate experiments. Samples were allowed to stand at room temperature for 30 min. Asterisks represent the significant differences between values with and without plasma at the same metHb ($P<0.01$). The small figure in the left bottom represents the change of BPA concentration as a function of time after addition of BPA to 4.2 mg/ml metHb solution. BPA values with different superscript letters in different times were significantly different ($P<0.01$).

4.2 mg/ml). Addition of 17% plasma inhibited the BPA decrease up to 4.2 mg/ml metHb, and completely inhibited up to 0.42 mg/ml metHb. Furthermore, addition of 23, 70 and 93% plasma did not bring any change in BPA recovery in the 4.2 ng/ml metHb solution.

To further confirm whether the decrease of BPA is caused by an existence of metHb in H-RBC, $NaNO_2$, a well known oxidative agent to change oxyHb to metHb, was added to the H-RBC. BPA concentration and metHb concentration were investigated at 30 min after addition of $NaNO_2$and BPA (Fig. 2).When 0, 0.04 and 0.4 mg/ml $NaNO_2$were added to water containing 300 ng/ml BPA, BPA recoveries were 98, 100 and 96%, respectively. However, when the same concentrations of $NaNO_2$ were added to H-RBC containing 300 ng/ml BPA, BPA recoveries were 56, 50 and 13%, and metHb concentrations were 0.8, 1.5 and 3.8 mg/ml, respectively.

Human normal physiological concentration of metHb is considered to be below 1–2% of total Hb. As adult total Hb is reported to be 0.15 g/ml in blood, metHb concentration is estimated to be 1.5–3 mg/ml in blood. Since fresh human H-RBC (diluted RBC to 5 times with water) is considered to contain less than 1.2 mg/ml metHb, it is necessary to dilute H-RBC at least a further 3 times with water to adjust the metHb concentration below 0.42 mg/ml, and to add more than 17% plasma or serum, for accurate total BPA determination (including MetHb-binding BPA) in RBC. Then, the recovery of BPA in human H-RBC with 20 ng/ml BPA added was investigated. After diluting H-RBC 3 times with water, human serum was added at a concentration of 20% to the diluted H-RBC and allowed to stand at room temperature. Recoveries of BPA in the human H-RBC at 0 (without serum) 1, 2 and 24 h after addition of serum were 10, 68, 69 and 79%, respectively.

The effect of hemolysis in serum on the BPA value was investigated. BPA concentration in non-hemolytic human sera containing 40 ng/ml BPA as a function of added metHb did not change significantly up to 2.5 mg/ml metHb. The recoveries of BPA in hemolytic human sera with 50 ng/ml added are shown in Table 3: they were 92.8% for non-hemolytic serum of a yellow color, 93.2% for pinkish serum with $\Delta OD=0.12$ (540–560 nm) for oxyHb and 94.8% for reddish serum with $\Delta OD=0.28$ for oxyHb. MetHb concentrations in three kinds of sera were below 0.05 mg/ml.

4. Discussion

HPLC-ED system has been established as a suit-

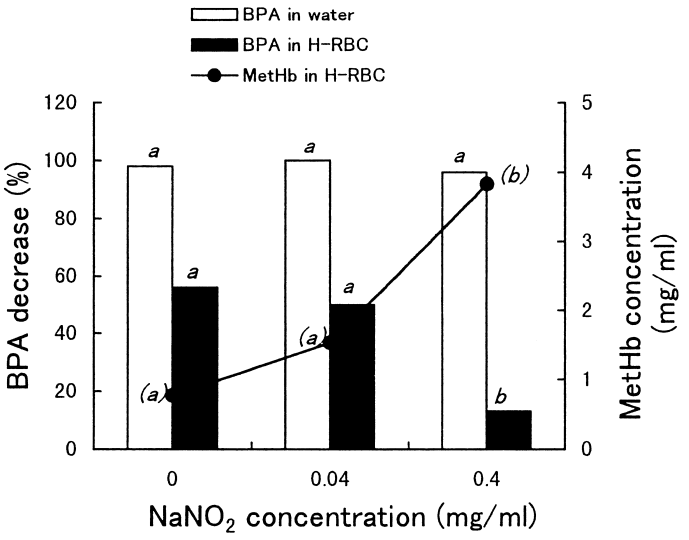

Fig. 2. Relationship between BPA and metHb concentrations in H-RBC treated with $NaNO_2$ Data represent the mean values of triplicates. Samples were allowed to stand at room temperature for 30 min. Values with different superscript letters of the same solutions were significantly different ($P<0.01$).

Table 3
Recoveries of BPA in hemolytic human sera[a]

Degree of hemolysis	Serum color	Serum oxyHb[b]	BPA recovery (%)[c]
None	Yellowish	0.09[x]	92.8[x]
Slight	Pinkish	0.12[y]	93.2[x]
Strong	Reddish	0.28[z]	94.8[x]

[a] Data represent the mean values of duplicates. Samples were allowed to stand at room temperature for 1 h.
[b] 50 ng/ml BPA was added.
[c] OxyHb values represent the ΔOD (540–560 nm).
Different superscript letters in the same column indicate a significant difference: $P<0.01$.

able analytical instrument for BPA determination in serum from the aspects of good reproducibility (C.V.=2.9), high sensitivity (the limit of detection, 0.2 ng ml^{-1}; $S/N=3$) similar to HPLC–MS [6]. A good correlation ($r=0.998$) of BPA values between both systems was observed. From the economic point of view, HPLC-ED is advantageous, since it is cheaper than HPLC–MS, and easy to handle.

To date, the dynamics of BPA in blood has not been well known, except for the assumption that BPA was not bound not to plasma proteins, such as sex steroid-binding proteins [4,10]. In the present study, BPA recovery did not change in sheep plasma allowed to stand for 1 day, which agreed with the previous assumption. However, BPA recovery changed significantly ($P<0.01$) in the presence of metHb, which showed that BPA determination in whole blood would be difficult, even when highly technical analytical methods such as GC–MS and LC–MS were used after extracting BPA by solid-phase extraction.

BPA recovery was significantly low ($P<0.01$) in the authentic metHb solution (ca. 4.2 mg/ml), which is close to physiological metHb concentration of sheep whole blood. This was observed a short time (within 10 min) after addition of BPA to metHb, suggesting that the decrease in BPA recovery would be related to the presence of metHb. Such a relationship between metHb and BPA recovery was also observed in the experiment using $NaNO_2$where the BPA recovery decreased drastically (13%, $P<0.01$) in the H-RBC containing almost the same metHb concentration. These results indicated that BPA determination using solid-phase extraction with water is impossible in whole blood or animal tissues containing abundant blood, because the BPA, after coming in contact with metHb derived from hemolyzed RBC, could not represent the real value.

Pedersen and Lindholst [14] reported that the BPA recovery was lower (49%) in the liver than in muscle (79%) of rainbow trout by HPLC–MS analysis based on microwave-assisted solvent extraction followed by solid-phase extraction. Miyakoda et al. [15] reported that the BPA recovery in the fetus whole body homogenate (87%) of the pregnant rat given considerably high concentrations of BPA (10 mg/kg), was lower than that in the maternal plasma (94%) by GC–MS analysis based on solid-phase extraction. Thus, the lower BPA recovery in fish liver or rat fetus whole body is considered to have arisen from the existence of metHb in situ. Moreover, the BPA concentrations in fetuses and maternal plasma were very low (<11 ng/ml for fetus and <34 ng/ml for maternal plasma) even after 1 h of BPA administration. The reason why BPA detected in vivo is very low could be attributed to the fact that metabolism of BPA is glucuronidation by UDT-glucuronosyltransferase mainly in maternal liver [16,17]. The influence of metHb on the BPA metabolism in vivo might be another cause of low BPA concentration in rats.

BPA concentration decreased abruptly within 10 min after BPA addition and thereafter recovered. Moreover, BPA concentration decreased in metHb solution added with plasma or serum and BPA, regardless of the order of addition of plasma or serum and BPA. This implies that the decrease of BPA recovery might be caused by the binding of BPA to metHb, not by metabolizing BPA. In the present study, 2.2 ng/ml BPA, which corresponded to the concentration of 18.3 ng/ml in RBC, was detected in H-RBC without BPA at 1 day after the incubation together with plasma. This might support the speculation that BPA could migrate from plasma to RBC and bind to metHb in RBC, and be released from metHb by reacting with plasma at 37°C for 1 day. When RBC in commercial sheep blood was separated from plasma which contained 599 ng/ml BPA [6], a high concentration of BPA (28 ng/ml without human serum and 54 ng/ml with 50% human serum) was detected in RBC (data not

shown), suggesting that BPA migrated from plasma to RBC and bound to metHb.

If BPA bind only to metHb in RBC, more than 90% BPA recovery is expected when 17% plasma is added to H-RBC containing less than 0.42% metHb. Therefore, the BPA recovery of 79% in human H-RBC suggests that BPA might be influenced by some other substances besides metHb in RBC and these substances are also suspected not to be influenced by addition of plasma. Thus, the determination of BPA in RBC separating from plasma is commendable.

Even strong hemolytic serum did not influence BPA values, indicating that hemolytic serum which physically occurred during blood collection contains trace amounts of metHb. It is necessary to further investigate the BPA recovery in the other components, such as white blood cells, to further assess the dynamics of BPA in whole blood.

5. Conclusion

Since it is very difficult to determine accurately the amount of BPA in whole blood due to the presence of metHb, it is necessary to separate plasma from RBC. A new method using HPLC-ED with solid-phase extraction devised for avoiding the influence of metHb is a sensitive and easy one for determining binding BPA in RBC.

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