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Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography

Junko Sajiki[a,*], Katsuhiro Takahashi[a], Jun Yonekubo[b]

[a]*The Public Health Laboratory of Chiba Prefecture, 666-2 Nitona-cho, Chuo-ku, Chiba City, Chiba 260-8715, Japan*
[b]*Nihon Waters K.K., 1-3-12 Kitashinagawa, Shinagawa-ku, Tokyo 140-0001, Japan*

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Abstract

The aim of this study was to establish an easy and accurate method for the determination of bisphenol-A (BPA) in the body liquid such as serum and urine. Two high-performance liquid chromatography (HPLC) systems, HPLC with electrochemical detector (ED), and HPLC with mass spectrometry (MS) using electrospray ionization (ESI) interface were used for the assay in the serum samples prepared with solid-phase extraction method. Water or EtOH at a concentration below 50% was suitable for the extraction of BPA from serum. The limit of detection of BPA was 0.2 ng ml^{-1} for the HPLC-ED method and 0.1 ng ml^{-1} for HPLC–MS. There was a good correlation between the data obtained by the two HPLC systems. BPA concentrations in healthy human serum were low (0–1.6 ng ml^{-1}). From various commercial fetal bovine serum and sheep plasma, however, significant amounts of BPA were detected. Since no BPA was detected from sheep plasma immediately after collection, the high amounts of BPA were considered to be caused by the handling of blood during the preparation of the products after blood collection. In vitro study showed that the amount of BPA leached from polycarbonate tube into sheep plasma were 40 times larger than those into water and the leached amount of BPA depended on the temperature (37°C>20°C>5°C).

Keywords: Bisphenol-A

1. Introduction

Since 1993, when Krishnan [1] documented that bisphenol-A (BPA) was released from polycarbonate flasks during autoclaving and had estrogenic activity, the effects of BPA on health has become a controversial issue. It was reported that significant amount of BPA was released in saliva from resin-based components, and sealant for dentistry [2–4]. There are many analytical reports concerning BPA in water and solvents from environmental water [5,6], baby food bottles [7], and plastic waste [8]. It is not known how much BPA contaminates living things including humans, mainly due to the lack of an appropriate method for the determination of BPA in biological samples.

To date, gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography equipped with ultraviolet (UV) or fluorescence detectors are commonly used for the determination of BPA in aqueous samples, while, HPLC–MS has been widely used for analyses of various

*Corresponding author. Tel.: +81-43-266-6723; fax: +81-43-265-5544.
E-mail address: sajiki@beige.ocn.ne.jp (J. Sajiki)

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substances in biological samples. Moreover, electrospray ionization (ESI) for the interfacing of MS has proven to desolvate and ionize fragile chemical species such as protein, peptides, nucleic acids and eicosanoids [9–11]. Yonekubo et al. [12] proposed direct analysis by HPLC–MS for the determination of BPA in water samples. They mentioned that ESI showed the highest sensitivity among three ionization methods (El, APCI and ESI). On the other hand, the electrochemical detector (ED) is now widely used to analyze phenol compounds in biological samples due to its high specificity [13,14]. Therefore, we established an easy and accurate method for the BPA determination in serum (or plasma) using two HPLC systems with ED and with ESI–MS. In this study, we analyzed BPA on several samples contained in different packages.

2. Experimental

2.1. Materials

Various fetal bovine (FB) serums and defibrinized sheep blood were purchased from Japanese pharmaceutical companies (Tokyo, Japan). BPA was purchased from Wako Pure Chemical (Tokyo, Japan). Polycarbonate (PC) centrifuge tubes (110 mm×16 mm l.D.) were purchased from Nalgenunc International (Tokyo, Japan). Freeze dried bovine serum albumin was purchased from Japanese animal industry (Chiba, Japan) and was dissolved in water to a final concentration of 5 g dl^{-1} (corresponding to that of human serum). Sheep blood were collected from leg vein of three sheep which were raised domestically. Human blood were collected from 21 (12 women, nine men) healthy Japanese adult volunteers aged 30 to 50. Plasma and serum were separated from red blood cells by centrifuging at 1000 *g* for 10 min. To avoid contamination of BPA, glass syringes and glass tubes were rinsed with 99% ethanol (EtOH) before use. Throughout the experiment, BPA-free water which was prepared using ODS-silica Sep-pak (Waters, Milford, MA USA) was used. ODS-silica Sep-pak (Waters, MA USA) or Oasis HLB (Waters) used for the solid-phase extraction of BPA were washed with 3.5 ml EtOH and 3.5 ml water. The eluent of Sep-pak and Oasis HLB with ethyl acetate was evaporated and checked for contamination before use. In every analysis, blank test using BPA free water was done to make sure there is no contamination.

2.2. Purification of BPA from plasma or serum

A 0.2–0.5 ml serum (or plasma) was diluted with BPA-free water to a final concentration 5 ml. The samples were applied to Sep-pak or Oasis, and polar lipids were removed from the column with 3.5 ml 15% EtOH and 3.5 ml petroleum ether used to remove nonpolar lipids after washing with 3.5 ml water. Finally, BPA was eluted with 3.5 ml ethyl acetate. The solvent was evaporated under N2. The residue was dissolved in 1 ml acetonitrile–water (40:60) solution. BPA standards (up to 500 ng ml^{-1}) were prepared with water using 1 μg ml^{-1} stock solution dissolved with 25% methanol (MeOH).

2.3. In vitro study on leaching of BPA in plasma and water from PC tube

Two ml of fresh sheep plasma free from BPA were divided into three PC centrifuge tubes and were allowed to stand at 5°C, 20°C and 37°C. A 0.2 ml plasma was taken from each tube for BPA assay every other day for 8 days. For the control, only water was used.

2.4. HPLC and MS condition

One of the HPLC systems, Model LC-10 AD (Shimadzu, Kyoto, Japan) with Shim-Pack VP-ODS column (150 mm×4.6 mm I.D., Shimadzu) and electrochemical detector (Coulochem II 5200A, ESA, MA, USA), was used for BPA analysis. The solvent system used was acetonitrile–water–phosphoric acid (40:60:0.2). Flow-rate and column temperature were 1.0 ml min^{-1} and 40°C. 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 time of BPA peaks with those of the authentic standards, and co-chromatography using authentic standards was also employed. For a complete identification, the BPA peaks eluted by HPLC

were confirmed using GC–MS (QP-5000, Shimadzu, Kyoto, Japan) after silylation with bis-(trimethylsilyl)trifluoroacetamide.

The other HPLC system was Alliance 2690 (Waters, MA, USA) with a Symmetry C_{18} column 3.5 μm (150 mm×2.1 mm I.D.) and a ZMD Z-spray mass spectrometer (Waters) using an ESI interface system [12] was used. The solvent system used was acetonitrile–water (40:60). Flow-rate and column temperature were 0.25 ml min^{-1} and 40°C, respectively. Analytical condition in the negative ion scanning modes of ESI were as follows: capillary voltage; 3.5 kV, cone voltage; 39 V for quantitative analysis, 59 V for quantitative (CID fragmentation) analysis, source block temperature; 130°C, desolvation temperature; 390°C. Injection volume was 20 μl. Data represented as means of duplicates.

3. Results

In the experiments, no contamination was observed during clean-up and analysis of BPA. Extractions of BPA in the serum and plasma samples with up to 50% water and EtOH showed the same recovery. However, with more than 50% EtOH, the recovery decreased (10% recovery at 99% EtOH). In an HPLC-ED system, the limit of detection was estimated 0.2 ng ml^{-1} (C.V.=2.9, S/N=3.0 (n=5)). When 10 ng ml^{-1} BPA was added to water, the values determined by HPLC-ED at day 0, 1, 2 and 5 were 9.6, 9.7, 9.3 and 9.8 ng ml^{-1}, respectively, which shows no significant variation. The BPA peaks in HPLC-ED were identified by GC–MS. In HPLC-ED analysis for BPA, there was no disturbance by other structurally similar compounds such as diethylstilbestrol, 17β-estradiol and 17α-ethinylestradiol. In an HPLC–MS system, the limit of detection was estimated to be 0.1 ng ml^{-1} (C.V.=7.0, S/N=3.1 (n=5)). The range of linearity of both methods was up to 100 ng ml^{-1}. The recovery of added BPA at 10 ng ml^{-1} to both human serum and sheep plasma was above 93% for both.

Fig. 1a shows the BPA peaks (illustrated in black) of the standard, FB serum and human serum monitored at pseudemolecular ion $(M-H)^-$ of BPA (m/z=227). Mass spectra of the HPLC peak corresponding to BPA in FB serum showed the same fragmentation pattern with the authentic BPA (Fig. 1b). The peaks of the samples were identified as BPA. There was a good correlation (Y=1.14X, r=0.998) between the BPA values in the same samples measured by the two systems. The averages and standard deviations of BPA concentrations in human serums were 0.33±0.54 ng ml^{-1} (0–1.6 ng ml^{-1}, n=12) for women and 0.59±0.21 ng ml^{-1} (0.38–1.0 ng ml^{-1}, n=9) for men. No BPA was detected in sheep plasma obtained immediately after the blood collection. Contamination during blood collection was negligible when EtOH treated glass apparatus was used. On the other hand, BPA concentrations in commercial FB serum, sheep plasma and bovine serum albumin analyzed by HPLC-ED and HPLC–MS are shown in Table 1. The values analyzed by HPLC–MS were slightly higher than those by HPLC-ED. BPA in all the serums kept in plastic bottles, A–D, were detected at high concentration. BPA concentration in plasma separated from defibrinized sheep blood showed the highest value in spite of having been kept in glass bottle. Significant amount of BPA was detected in a freeze dried bovine serum albumin kept in plastic container.

The amounts of BPA in sheep plasma free from BPA stored in PC centrifuge tubes increased significantly depending on temperature and time (Fig. 2). Leaching of BPA from PC tubes to plasma were much larger than those from PC to water under all temperatures. In particular, at day 8 after the start of the experiments, BPA concentration in plasma (333.5 ng ml^{-1}) allowed to stand at 37°C was 44.5 times higher than that in water (7.5 ng ml^{-1}) and the concentrations in plasma at 20°C and 37°C were 3.9 and 12.4 times higher than that at 5°C, respectively. Similar phenomenon was observed in human serum. The leaching amount of BPA (mean±SD) in human serum at day 4 and 8 allowed to stand in three individual PC tubes at 37°C were 112.2±8.0 ng ml^{-1} and 369.7±53.0 ng ml^{-1}, respectively.

4. Discussion

Here, a device for the easy purification of BPA from biological samples such as serum and plasma using Sep-pak and Oasis cartridges was carried out

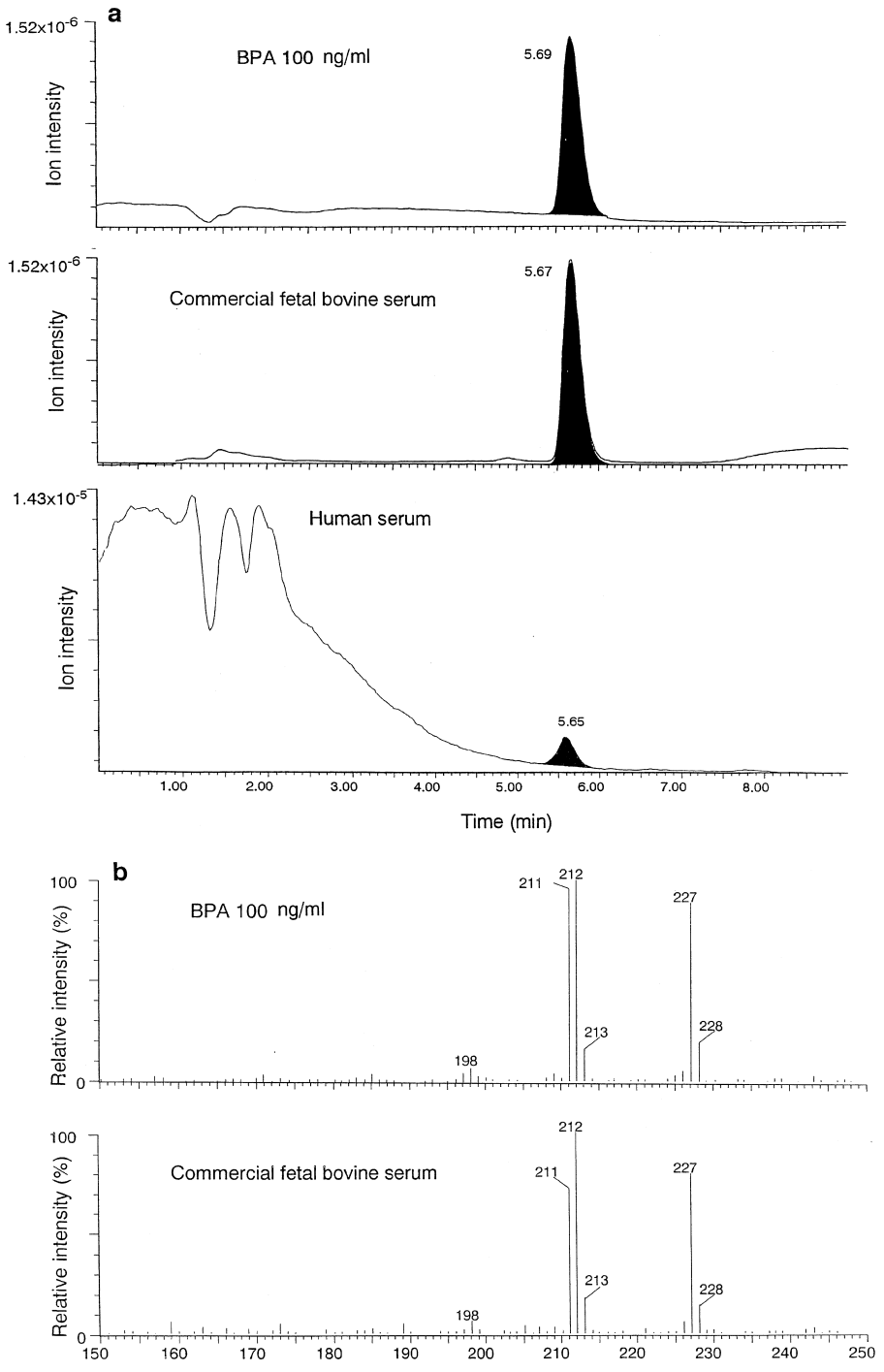

Fig. 1. Ion intensity of BPA in authentic standard, commercial fetal bovine serum and human serum detected by HPLC–MS using ESI (a) and mass spectrum of BPA peak in the standard and the fetal bovine serum at 59 V of cone voltage (b). Monitored using the pseudemolecular ion $(M-H)^-=227$ of BPA. Mobile phase, acetonitrile–water (40:60, v/v). A 50 μl volume of the samples dissolved in mobile phase was injected.

Table 1
BPA concentration of various commercial samples analyzed by HPLC-ED and HPLC–MS methods[a]

Sample	Storage condition	Packaging	BPA concentration (ng ml^{-1})	
			HPLC-ED	HPLC–MS
Fetal bovine serum				
A	Deep freeze	Plastic bottle	24	20
B	Deep freeze	Plastic bottle	39	34
C	Deep freeze	Plastic bottle	154	135
D	Deep freeze	Plastic bottle	236	217
Defibrinized sheep blood	Cool below 5°C	Glass bottle	599[b]	–
Bovine serum albumine	Freeze dry	Plastic bottle	42[c]	41[c]

[a] Data are represented as mean of duplicates.
[b] In plasma.
[c] In water solution of 5 g dl^{-1}.

and there was no difference between the results obtained using both cartridge types. Both HPLC-ED and HPLC–MS methods were found to be suitable for analyses of aqueous biological samples such as serum and plasma from the aspects of good reproducibility, high accuracy, and easiness to handle.

It is considered that BPA is easily leached from the dental composites and sealant into saliva due to its moderately soluble in water [5]. In this study, FB serums kept in plastic bottles contained high concentrations of BPA (24–236 ng ml^{-1}). Surprisingly, the highest amount of BPA (599 ng ml^{-1}) was detected in plasma prepared from commercial sheep blood kept in glass bottle. We suspect that contamination by BPA at high levels in blood could be caused by the handling of blood after collection. For example, blood might have been stored in plastic containers during the preparation procedure such as defibrination before dividing into glass bottles. This is explained by the fact that BPA was not detected in the plasma which was separated immediately after the collection from domestically raised sheep and BPA was not detected for ten days when the plasma was allowed to stand at 20°C. Moreover, consider-

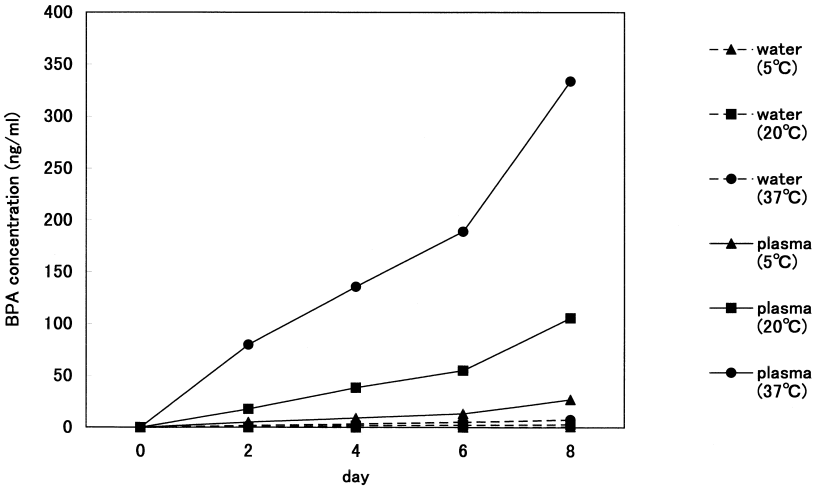

Fig. 2. BPA concentrations in sheep plasma and water leached from PC tube as a function of ambient temperature and time. Aliquot was taken from the PC tubes allowed to stand at different ambient temperatures every assay day. Measured by HPLC-ED method.

able amounts of BPA were leached in plasma from PC tubes even at 5°C, and the concentration was more than 40 times higher than in water at 37°C, the body temperature. These results suggest that BPA might be highly soluble in body fluid such as serum and saliva. There is a question whether leaching of BPA in the body fluid could be a result of enzymatic reactions. An extra experiment using human serum and water allowed to stand at 60°C for 3 days resulted in a significant increase (8 times higher) in the leaching amount of BPA than those in both samples at 37°C. The results suggest that the leaching rate of BPA could be relative to the temperature and the concentration of components in the body fluid such as lipids. The difference in BPA concentrations among the FB serums might be caused by the materials of the container and/or the length and temperature during storage. Recently, it was reported that low concentrations of BPA (2–20 ng ml^{-1}) caused disorders in murine fetal reproductive organs [15–17] and stimulation of prolactin released from primary anterior pituitary cells [18]. As these commercial serums are supplied in many laboratories producing vaccine and dealing with cultured cells and tissues for varied purposes, it is necessary to further investigate a possible adverse effect via serum.

As for the metabolism of BPA, a few evidences have been reported. In vivo, BPA was converted to a hydroxylated bisphenol-A [19]. Atkinson and Roy [20] observed that an incubation of BPA with rat liver DNA in the presence of peroxidase activation system, which produced DNA adducts such as bisphenol *o*-quinone-DNA in vitro. They indicated that abundant BPA might be chemically converted to bisphenol *o*-quinone and further to DNA binding metabolites. In this study, very low concentrations of BPA (0–1.6 ng ml^{-1}) in healthy human serums may be caused by this metabolism of BPA.

The leaching rate of BPA to sheep plasma depended on temperature, especially in the body temperature range where the BPA released to body fluid such as plasma and saliva would be enhanced. Recently, a new resin component for dental materials with low toxicity has been developed [21]. Thus, it is necessary to further investigate the migration dose of BPA in vivo from dental and surgical devices widely used [22].

5. Conclusion

We concluded that the two HPLC systems used along with solid-phase extraction were effective in detecting low concentrations of BPA in biological samples. The advantages and disadvantages are different between two systems. The HPLC-ED is easy to handle and maintenance is cheap, but it can not identify BPA in samples. On the other hand, it is possible to identify BPA with HPLC–MS but it is expensive to maintain. Therefore, these two methods should be used together for a complete identification.

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