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A new competitive enzyme-linked immunosorbent assay (ELISA) for determination of estrogenic bisphenols

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Abstract

Bisphenol A and other hydroxylated diphenylalkanes (generally known as bisphenols) have been identified as potential estrogenic substances. In this paper, a specific polyclonal antibody was produced against these compounds by immunization of rabbits with a conjugate of 4,4-bis (4-hydroxyphenyl) valeric acid and bovine serum albumin (BHPVA-BSA). The polyclonal antibody showed specific recognition of the bisphenol structure, while the cross reactions of other common phenolic compounds such as phenol, hydroquinol and p-hydroxybenzoic acid were all lower than 1%. The linear range of bisphenol A calibration curve was 1–10 000 ng ml^{-1}. Real water samples and mouse serum samples were spiked with known amount of bisphenol A and measured by the competitive ELISA. Recoveries were between 92 and 105%. The detection limits were found to be 0.1 ng ml^{-1} for real water samples and 2 ng ml^{-1} for serum samples. The method is very useful for monitoring bisphenol compounds in environmental and biological samples.

Keywords: Bisphenol A; Polyclonal antibody; Competitive ELISA; Water sample; Serum sample

1. Introduction

Since Krishnan [1] reported in 1993 that bisphenol A (BPA) released from flasks made of polycarbonates had weak estrogenic property, many researches have been done to test and verify its effect on animals and human beings [2,3]. Perez et al. [4] identified that besides BPA, some other related hydroxylated diphenylalkanes are also estrogenic. Since these compounds are widely used as monomers of various plastics such as epoxy

Abbreviations: BHPVA, 4,4-bis(4-hydroxyphenyl) valeric acid; BPA, bisphenol A; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide · HCl; FCA, Freund's complete adjuvant; FIC, Freund's incomplete adjuvant; HRP, horseradish peroxidase; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; PBS, phosphate buffered saline; PBST, 0.01 mol l^{-1} PBS containing 0.05% Tween 20; PP, phenolphthalein; SAS, saturated ammonium sulfate; TMB, 3,3′,5,5′-tetramethylbenzidine.

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resin and polycarbonates, which are largely used in food packaging and containers, potential exposure of human beings to them is becoming a significant issue.

So far, many chromatographic methods combined with SPE and other chemical analytical techniques have been established for analysis of these compounds in miscellaneous samples [5–8]. The common drawbacks of these methods are the time-consuming sample pretreatment steps. For GC–MS detection, a tedious derivatization process is often required. Seifert et al. [9] has developed a method based on receptor affinity chromatography coupled to LC–MS–MS. Although the receptor affinity chromatography is excellent for screening the estrogenic substances, it is rather difficult to obtain the human estrogen receptor. Regarding of the biological activity of bisphenols, we thought an antibody–antigen recognition system might be a specific and sensitive tool to detect them. There have been some reports concerning the production of antibodies against BPA [10,11]. But in their methods, one of the two hydroxy groups of BPA was carboxylated so as to conjugate BSA. Thus part of the structural characteristics of BPA was lost in the synthesized antigen. In this paper, we chose a commercially available reagent 4,4-bis(4-hydroxyphenyl) valeric acid (BHPVA) to prepare the complete antigen and produced a specific polyclonal antibody against bisphenol compounds. High titer of antiserum was obtained and a quantitative indirect ELISA was established for sensitive and specific determination of the concerned estrogenic bisphenol compounds.

2. Experimental

2.1. Materials

BHPVA was purchased from Aldrich Chemical Co. (USA). BPA and phenolphthalein (PP) were obtained from Beijing Haidian Xinxing Reagents Factory and Beijing Chemicals Co., respectively. Bovine serum albumin (BSA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide · HCl (EDC), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIC), ovalbumin (OVA) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Beijing Biotinge Biotech Co. (China).

Stock solutions of BPA, BHPVA, PP, phenol, hydroquniol and p-hydroxybenzoic acid (1.0 mg ml^{-1}) were prepared by dissolving 10.0 mg of the compound in 10.0 ml of dimethyl sulfoxide (DMSO). The working solutions were made freshly before use by diluting the stock solutions with 0.01 mol l^{-1} PBS.

Microwell plates were purchased from Nunc, Denmark and a Bio-Rad Model 550 Microplate Reader was used to measure the optical density of the ELISA results. A Cary 1E UV–Vis Spectrometer (Varian) was used to measure the absorbance of the protein solution.

The real water samples were obtained from a lake (The Weiming Lake of Peking University). The mouse serum was collected from normal BALB/c mice raised in the Center of Laboratory Animals in Peking University.

2.2. Preparation of the complete antigen

NHS and EDC were used to activate the BHPVA. The molar ratios of BHPVA, NHS and EDC were 1:1.25:1.25. Typically, 7.0 mg of BHPVA, 3.5 mg of NHS and 5.9 mg of EDC were weighed and dissolved in 1 ml DMSO in a 10 ml glass vial and the reaction was carried out for 2 h under magnetic stirring. The obtained solution was referred to as solution A. BSA solution was prepared by dissolving 45 mg of BSA in 4 ml of 0.1 mol l^{-1} $NaHCO_3$ (pH adjusted to 7.0 by adding HCl). To this solution, solution A was added slowly in droplets under stirring. The reaction was performed for 2 h and the obtained solution was dialyzed against 0.01 mol l^{-1} phosphate buffered saline (PBS) at 4 °C for 24 h. After six changes of the PBS, the solution was dialyzed against pure water for 48 h with eight changes of water. Finally, the solution was lyophilized and the white crystal obtained was stored at −20 °C. The molar ratio of BHPVA-

BSA conjugate was measured using a Coomassie Brilliant Blue spectrophotometric method [12]. The BHPVA-OVA conjugate was prepared in the same way as the BHPVA-BSA.

2.3. Production and purification of the polyclonal antibody

Three New Zealand white rabbits (each weighs 2–3 kg) raised in the farmhouse were used for the immunization. For each rabbit, a total of four injections of 4 mg of BHPVA-BSA conjugate in Freund's adjuvant were performed at days 0, 11, 23, and 34 before final bleeding at day 45. The first injection of 1.5 mg of the antigen was emulsified with FCA. The later three booster injections were emulsified with FIC and contained 0.5, 1 and 1mg of the antigen, respectively. The titre of antiserum was determined with ELISA using BHPVA-OVA coated onto the microplate.

The obtained antibody was purified according to a modified caprylic acid-saturated ammonium sulfate (SAS) method [13]. To 2 ml of antiserum diluted with 6 ml of acetate buffer (0.06 mol l^{-1}, pH 4.8), 200 μl of caprylic acid was added dropwise under gently magnetic stirring. The formed turbid solution was slowly stirred for 30 min and then centrifuged at 12 000 rpm for 30 min at room temperature (20 °C). The precipitates were discarded and 0.1 mol l^{-1} PBS was added to the supernatant at a volume ratio of 1:10. The solution was adjusted to pH 7.4 with 1.0 mol l^{-1} NaOH and cooled to 4 °C. To this solution SAS (pH adjusted to 7.4) of the same volume was added dropwise under magnetic stirring. The obtained solution was allowed to stand for 2 h and then centrifuged at 12 000 rpm for 30 min at 4 °C. The supernatant was discarded and the precipitates were dissolved in 2 ml of 0.01 mol l^{-1} pH 7.4 PBS. The solution was dialyzed against 0.01 mol l^{-1} PBS until no sulfate ion could be detected in the dialysis solution and then stored at −20 °C.

2.4. Titration level assessment of antiserum and purified antibodies

The optimum concentration of BHPVA-OVA and the titers of antiserum and purified antibodies were determined by a chequerboard method [14]. In brief, a 96-well microtiter plate was coated with 100 μl of BHPVA-OVA at different concentrations in 50 mmol l^{-1} carbonate buffer (pH 9.6) overnight at 4 °C. Then the wells were washed three times with 370 μl of 0.01 mol l^{-1} PBS containing 0.05% Tween 20 (PBST) and blocked using 200 μl of 0.8% gelatin in PBS for 2 h at 37 °C. After washing three times with PBST, the wells were incubated with 100 μl of 10-fold serially diluted antiserum or purified antibodies for 1 h at 37 °C. To the washed wells 100 μl of 1:1000 diluted HRP-conjugated goat anti-rabbit IgG was added and incubated for 1 h at 37 °C. After washing three times with PBST and then twice with pure water, the wells were filled with 100 μl of 60 μg ml^{-1} TMB in DMSO dissolved in citrate phosphate buffer (pH 6.0) containing 450 ppm of H_2O_2. After incubation for 15 min at room temperature, 50 μl of 2 mol l^{-1} H_2SO_4 was added to stop the enzyme reaction and the absorbance of each well was determined with the Bio-Rad microplate reader at 450 nm.

2.5. Competitive ELISA system for specificity study and quantitative determination of BPA

The specificity of the polyclonal antibody was investigated by testing BHPVA, BPA, PP and three commonly existing phenolic compounds, including phenol, hydroquinol and p-hydroxybenzoic acid. The microtiter plate was coated with 100 μl of 12.0 mg l^{-1} BHPVA-OVA and blocked in the same way as above. Standard solutions of known concentration (0–100 000 μg ml^{-1}; 50 μl per well) of one of the competitive compounds were added together with 50 μl of the 1:5000 diluted purified antibody solution and incubated for 1 h at 37 °C. The following steps were similar to the above-described procedure. The wells were washed three times after each incubation step with 370 μl of PBST.

The recoveries of the assay were determined by spiking the water samples and serum samples with known amount of BPA and comparing with the spiked standard diluent controls.

3. Results and discussion

Since bisphenols are not capable of initiating an immune response themselves, they need to be conjugated with a protein to form a complete antigen. In the previous reported ELISA method [10], the antigen was synthesized by activating one of the hydroxy groups of BPA and then coupling with the carrier protein. In such a conjugate, part of the characteristics of the bisphenol structure was lost, resulting in a low selectivity for most of the measured compounds containing bisphenolic structure (cross-reactivity lower than 1%), which have been confirmed to be a group of estrogenic substances. In this paper, BHPVA was used to prepare the antigen due to its relatively long carbon chain with a reactive carboxyl group on the end. The conjugation took place between the amino group of BSA and the carboxyl group of BHPVA, thus the structure of interest was favorably exposed (Fig. 1). The molar ratio of BHPVA/BSA was found to be 20:1.

Fig. 2 showed the chequerboard titration results for the antiserum. Based on the definition of titer as the antiserum dilution required to bind 50% of a small, given amount of labeled antigen, the titers of the antiserum and purified antibody were found to be 1:50 000 and 1:10 000, respectively, and the optimal concentration for BHPVA-OVA as the coating ligand was 12.0 μg ml^{-1}.

In this study, three compounds containing the structure of two phenolic rings joined together through a bridge carbon (BPA, BHPVA and PP) and three simple phenolic compounds (phenol, hydroxyquinol and p-hydroxybenzoic acid) were involved. Their chemical structures were shown in Fig. 3. The Dose–response curves of the selected

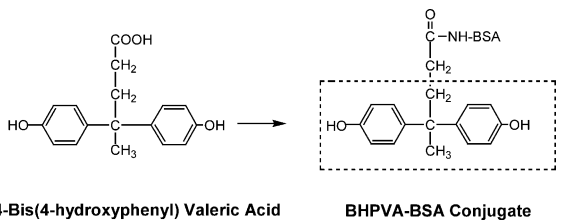

Fig. 1. Synthesis of the complete antigen of bisphenol compounds.

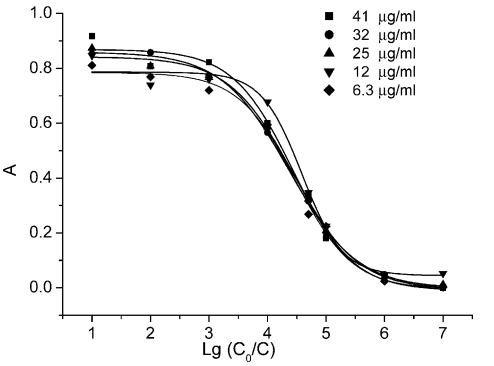

Fig. 2. Chequerboard titration results of the antiserum. The microtiter plate was coated with 100 μl of BHPVA-OVA at different concentrations (41, 32, 25, 12 and 6.3 μg ml^{-1}) in 50 mmol l^{-1} pH 9.6 carbonate buffer. For each concentration level of the coated antigen, the antiserum was 10-fold serially diluted and added to the well. The titer was determined as the antiserum dilution corresponding to 50% of the maximum absorbance.

compounds were shown in Fig. 4. It can be clearly seen that with the increasing of the concentration of the competitive compounds, the antibody–antigen reaction was significantly inhibited by the hapten (BHPVA), BPA, and PP, while the other three structurally similar compounds have little influence on the reaction. According to the 50% displacement method [15], the cross reactions of BPA and PP with BHPVA were 53 and 23%, respectively, while the cross reactions of the other three compounds were all much lower than 1%. The common part of the structures of BPA, BHPVA and PP are the two phenolic rings bridged

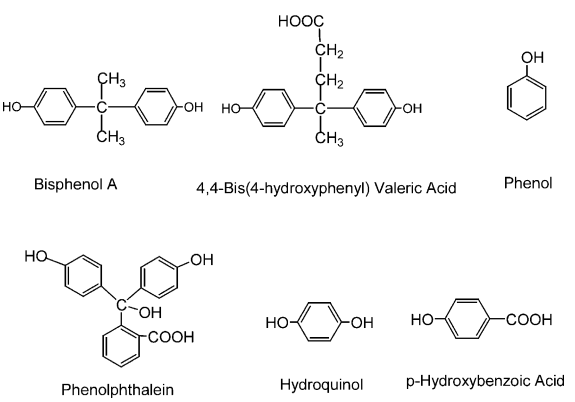

Fig. 3. The chemical structures of the six selected compounds.

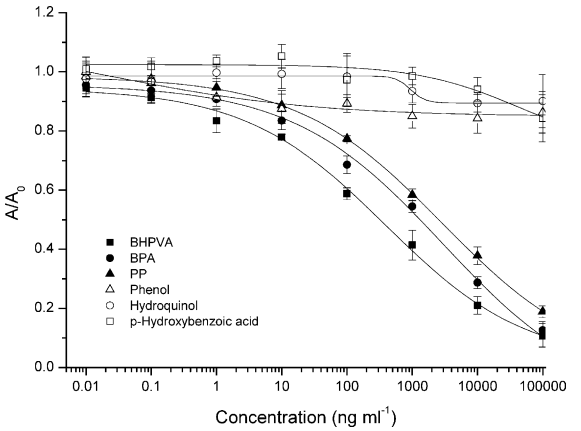

Fig. 4. Dose–response curves of the selected compounds based on competitive ELISA.

through a carbon atom. Thus it was demonstrated that the produced polyclonal antibody specifically recognizes such a bisphenolic group.

It's remarkable that PP, a most commonly used acid-base indicator and an over-the-counter laxation, has been found recently to exhibit carcinogenic activity [16]. From our experimental results, it shows notable cross-reactivity with the anti-bisphenol polyclonal antibody. Additional studies may be required to further investigate the estrogenic potentiality of this compound.

The logit-log algorithm was used to establish the linear regression [14]. The linear range was found to be between 1 and 10 000 ng ml^{-1}. The equation was $\ln(A/(A_0 - A)) = 2.354 - 0.782 \log C_{BPA}$ ($R^2 = 0.990$, $n = 5$). No significant matrix effect was observed when the ELISA was applied to measure the spiked real water samples. But for the serum samples, a 1:10 dilution with 0.01 mol l^{-1} pH 7.4 PBS was necessary prior to addition to the well to obtain quantitative recoveries. The recovery results were shown in Table 1. The detection limits were found to be 0.1 ng ml^{-1} for real water samples and 2 ng ml^{-1} for serum samples. The within-assay coefficient of variation was less than 8% and the between-assay coefficient of variation was less than 15%.

Compared with the existing chromatographic methods, though the established ELISA is not as sensitive as GC–MS combined with solid phase extraction and derivatization steps (limit of detection 4–6 ng l^{-1}) [5], it offers the advantages of simplicity, high sample turn over, small sample amount and potentiality to be developed into test kits. In addition to the higher selectivity and more readily available reagents, the present ELISA is also more sensitive than the previously published ELISA [10] by which the limit of detection for BPA was 5 ng ml^{-1}.

Table 1
Recovery of BPA in the real water and serum samples by the established ELISA

BPA pg per well	Recovery (%)[a]	
	Water samples	Serum samples
10	103 ± 3	–
100	105 ± 4	104 ± 7
1000	92 ± 7	96 ± 5

[a] The recovery has been determined by spiking BPA in real water and mice serum samples and comparing with spiked standard diluent controls. The data was expressed as mean ± S.D. of ten experiments.

Since the human estrogenic receptor is relatively difficult to obtain, the proposed immunoassay system may be a practical alternative for screening the concerned estrogenic bisphenolic compounds.

In conclusion, the presented ELISA is a very useful tool for detecting bisphenol compounds represented by BPA in environmental water and biological samples. It's also a promising technique for monitoring the bisphenol level in aqueous food samples.

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