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Development and comparison of two competitive ELISAs for the detection of bisphenol A in human urine

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Bisphenol A (BPA) is widely used to manufacture polycarbonate plastics and epoxy resins, which are in widespread use in China. Concerns about potential health impacts from exposure to BPA among the general population are increasing day by day. A sensitive and specific antibody was prepared to develop and compare direct and indirect competitive enzyme-linked immunosorbent assays (ELISAs) for the determination of BPA in human urine. The IC_{50} value and limit of detection (LOD) for the direct competitive ELISA were 5.5 ng mL^{-1} and 0.03 ng mL^{-1} respectively whereas, for the indirect competitive ELISA, the IC_{50} value and LOD were 7.0 ng $mL^{-1}$and 0.08 ng mL^{-1}, respectively. We found that the direct competitive ELISA was more specific and sensitive than the indirect competitive ELISA. The established immunoassays were also applied in the determination of BPA in human urine samples. The results from fortified samples and real samples indicated that the direct competitive ELISA was more sensitive and reliable for the measurement of BPA in human urine as compared to the indirect competitive ELISA.

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Introduction

Exposure to environmental pollutants is considered to be one of the major global health concerns.[1–4] There are numerous pollutants that may influence the development of the immune system and the weakening of the immune system may be the underlying cause of the pathophysiology of many chronic diseases.[5–8] Among these environmental pollutants, the most important are the plastics that have their widespread use worldwide especially in China. The most important component of these plastics, bisphenol A (BPA), is an industrial organic compound which is produced in large quantities primarily for use in the manufacturing of polycarbonate plastics and epoxy resins.[9,10] Polycarbonates are used in containers that store food and beverages and epoxy resins are used as protective coatings on food cans because of their durability, and because of the fact that they adhere well to metals and are highly resistant to chemicals.[11] In addition BPA is used in several other items, that we come into contact with daily at home and in the workplace, namely electronic equipment, automobiles, safety equipment, recycled materials, CDs and DVDs.[12] BPA leaches out from polycarbonate plastics and epoxy resins that are in contact with food and beverages, and as a result, BPA is ingested by the consumers of these products.[13] Several cross-sectional studies have shown that measureable levels of BPA have been detected in individuals.[9,14–19] Human exposure to BPA is usually from drinking water, medical and dental procedures, commercial transactions, and household dust.[20–22] Following ingestion, BPA is metabolized to form glucuronide-conjugated BPA and is excreted from the body *via* urination.[23] Other than ingestion of BPA, humans may also be exposed to BPA *via* air and skin.[24,25] Increased exposure to BPA in humans may cause prenatal and neonatal abnormalities,[26] changes in the uterus and ovaries,[27,28] alterations in brain sexual dimorphisms,[29,30] altered glucose homeostasis[31] and altered sociosexual behavior.[32]

Several methodologies have been developed for the detection of BPA but these methodologies require sample pre-treatment, are time consuming and very costly. There are many advantages of using ELISA as there is high specific interaction between antibodies and antigens, the sensitivity is high, and high throughput is achieved with low cost. For the determination of low molecular weight environmental contaminants, competitive ELISA is one of the most suitable methods.[33] In our present study, we have developed two highly sensitive and specific competitive (direct and indirect) ELISAs for the detection of BPA in human urine samples.

Materials and methods

Chemicals and reagents

BPA, 4,4-bis (4-hydroxyphenyl) valeric acid (BHPVA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) and horseradish peroxidase (HRP)

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were purchased from Aladdin (Shanghai, China). Tween-20, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), bovine serum albumin (BSA), ovalbumin (OVA) and β-glucuronidase were purchased from Sigma (Luis, Mo. 63178, USA). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sangon Biotech (Shanghai, China). HRP labeled goat anti-rabbit lgG conjugate (HRP-GaR lgG) was obtained from Boshide (Wuhan, China). New Zealand rabbits were purchased from the Experimental Animal Center of Zhejiang University (Hangzhou, China). Human urine samples were collected from 24 volunteers in Zhejiang Province with informed consent from all the participants. Blank urine sample was collected from a volunteer with a urine sample negative for BPA as detected by HPLC and GC-MS. Before the measurement, all the urine samples were treated with β-glucuronidase to release free BPA. All the experiments on animals were performed according to the ethics committee of the Experimental Animal Center of Zhejiang University.

Instruments

Microtiter plates were purchased from CoStar (CoStar Inc., Cambridge, MA, USA). The microplate reader was an R640 from Bio-Rad (Hercules, CA, USA). The UV spectrophotometer was an Hitachi U-3010 from Meitian Keji Co., Ltd. (Beijing, China).

Buffers and solutions

Carbonate buffer (0.1 M, pH 9.6) was chosen as the coating buffer. Phosphate-buffered saline (PBS) (10 mM L^{-1} sodium phosphate, 137 mM L^{-1} NaCl, 2.7 mM L^{-1} KCl [pH 7.4]) was used for the dilution of antibodies and preparation of standard solutions. PBST was used for washing and was prepared by adding 0.05% Tween-20 (v/v) to the PBS. Blocking buffer was prepared by adding 5% skimmed milk (w/v) to PBST. The substrate buffer was 0.1 M citrate (pH 5.5). The substrate solution was prepared by adding 125 μL of TMB solution and 2 μL 30% H_2O_2 to 10 mL of substrate buffer. 1 M H_2SO_4 was used to stop the enzyme reaction. Standard solutions of BPA were prepared from a stock solution of 1 mg mL^{-1} in methanol.

BPA hapten derivatives for conjugation with proteins

The synthetic pathways for haptens used for conjugation with proteins are presented in Scheme 1. The procedures for the synthesis of haptens (BPA-HS1 and BPA-HS2) were designed according to the procedures described previously with some modifications[34,35] and are shown as follows.

BPA-HS1, {4-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-phenoxy}-acetic acid. A mixture of BPA (1.14 g, 5 mM), ethyl bromoacetate (550 μL, 5 mM) and anhydrous potassium carbonate (828 mg, 6 mM) was dissolved in 20 mL dry acetone in a conical flask. The mixture was then heated to 70 °C and refluxed with stirring in a drying tube for 8 hours. The reaction mixture was cooled and then the solvent was removed by spin evaporation *in vacuo*. The resulting mixture was dissolved in 10 mL methanol and 5 mL sodium hydroxide (10%, w/v) and heated under reflux for 30 minutes. The pH of the reaction mixture was adjusted to 2.0 with HCl. The reaction mixture was washed with acetic ether (3 × 100 mL). The acetic ether phase was collected and dried over anhydrous sodium sulfate and the solvent was removed by evaporation. The residue was crude BPA-HS1, which was further purified by silica gel column chromatography.

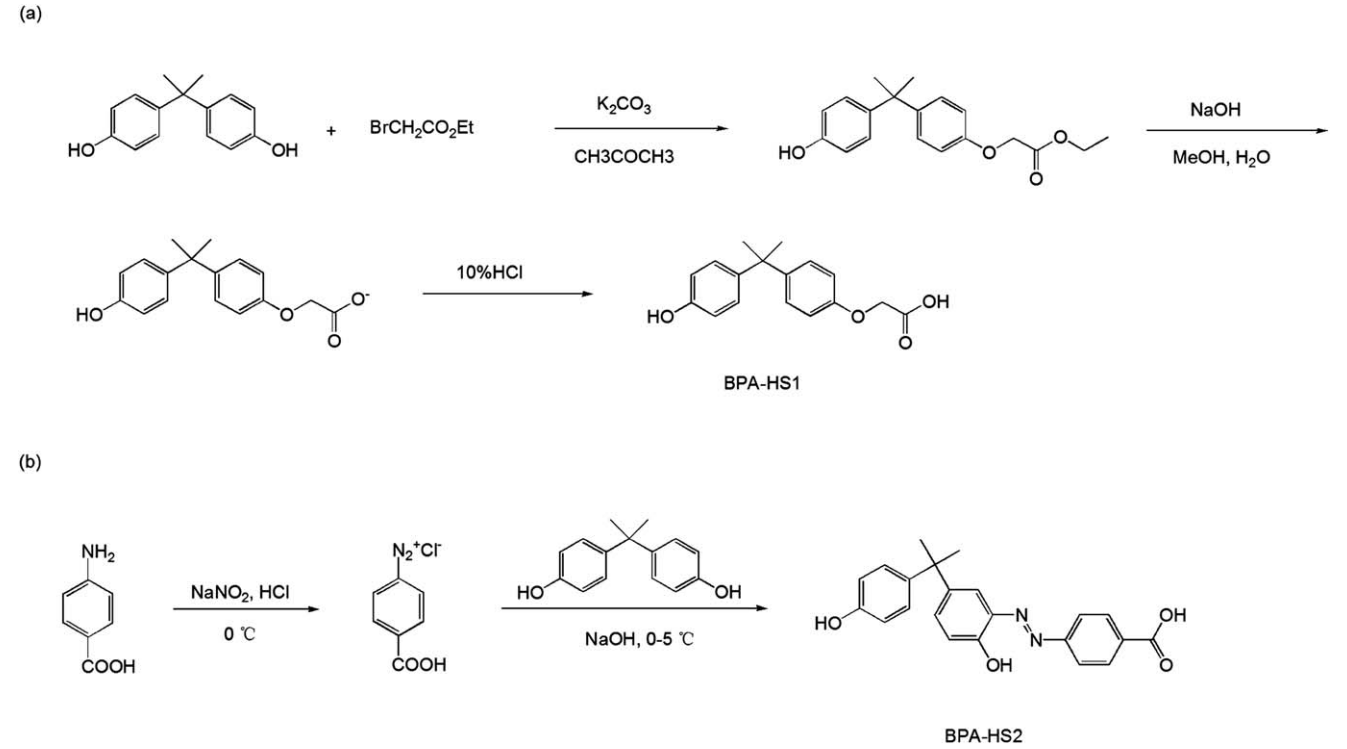
BPA-HS2, 4-{2-hydroxy-5-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-phenylazo}-benzoic acid. Para-aminobenzoicacid (PABA) (137 mg, 1 mM) was dissolved in 10 mL HCl (1 M). To it 250 μL $NaNO_2$ was added at 0 °C. The solution was added drop by drop into a solution of BPA (228 mg, 1 mM) in 20 mL NaOH (25% m/v). The mixture was stirred for 1 hour at °C. The solid obtained was filtered, washed with methanol and dried to get BPA-HS2.

Preparation of BSA and OVA conjugates

BPA-HS1, BPA-HS2 and BHPVA were conjugated to BSA and OVA by the active ester method.[33] The COOH groups on the hapten derivatives were activated with EDC and NHS to produce active esters, which were then reacted with the amine groups on BSA to form amide bonds. Briefly, BPA-HS1 (4 mg, 14 mM) was dissolved in 0.1 mL anhydrous DMF, NHS (1.72 mg, 15 mM) and EDC (2.88 mg, 15 mM) were then added. This activation reaction was carried out for 2 h at 37 °C with continuous stirring. BPA-HS2 (5 mg, 13 mM) was dissolved in 0.1 mL anhydrous DMF, NHS (1.61 mg, 14 mM) and EDC (2.68 mg, 14 mM) were then added. This activation reaction was carried out for 2 h at 37 °C with continuous stirring. BHPVA (4 mg, 14 mM) was dissolved in 0.1 mL anhydrous DMF, NHS (1.72 mg, 15 mM) and EDC (2.88 mg, 15 mM) were then added. The activation reaction was carried out for 2 h at 37 °C with continuous stirring. After centrifugation, 20 μL supernatant of each activated BPA hapten derivative was added very slowly to a BSA solution (3 mg BSA dissolved in 10 mL PBS), 10 μL supernatant of activated BPA-HS1 was added very slowly to an OVA solution (3 mg OVA dissolved in 10 mL PBS). The mixtures were stirred overnight at 4 °C to complete the conjugation reaction. The final solution was then dialyzed against phosphate buffer (0.1 mol L^{-1}, pH7.4) for 5 days (with three changes of buffer per day).

Preparation of HRP conjugate

The HRP conjugate was synthesized by the periodate method using coating antigen and HRP.[36] In brief, 2 mg HRP was dissolved in 0.3 mL deionized water, which was added to 0.1 M sodium periodate and stirred constantly at 37 °C for 30 min. At this point, the colour of the reaction solution was dark green. Then, 0.2 mL ethylene glycol (1%) was added to react with the extra sodium periodate. The colour of the reaction solution gradually turned to brown. After 10 minutes, 1 mg BPA-OVA was, dissolved in 0.5 mL deionized water and added slowly to the oxidized HRP solution. The mixture was centrifuged and the supernatant was kept in a dialysis bag against carbonate buffer (0.05 mol L^{-1}, pH 9.6) at 4 °C overnight. The next day, the solution from the dialysis bag was removed carefully and 0.1 mL of 4 mg mL^{-1} sodium cyanoborohydride was added to the solution, followed by reaction at 4 °C for 2 h. Finally, the mixture was dialyzed against phosphate buffer (0.1 mol L^{-1}, pH 7.4) at 4 °C for 2 days (with three changes of buffer per day). The



Scheme 1 (a) Synthesis of BPA hapten 1 (BPA-HS1). (b) Synthesis of BPA hapten 2 (BPA-HS2).

synthesized BPA–HRP conjugate was mixed with glycerol (1 : 1) and stored in darkness at −20 °C till further analysis.

Antibody production

Immunization of seven New Zealand white rabbits was carried out by subcutaneous inoculation. BPA-HS1-BSA, BPA-HS2-BSA and BHPVA-BSA immunogens were emulsified in FCA for primary immunization. Booster doses of three immunogens were emulsified in FIA. Different schemes (Table 1) were used for the immunization. In scheme A, 0.1 mg immunogen was used in every injection, booster doses of immunogens were injected at two-week intervals. In scheme B, 0.1 mg immunogen was used in every injection, booster doses of immunogens were injected at four-week intervals. In scheme C, 0.05 mg immunogen was used in every injection, booster doses of immunogens were injected at eight-week intervals. Blood samples were taken 10 days after each immunization. The antisera were obtained from the blood by centrifugation at 3000 RPM for 15 min and then stored at −20 °C for further analysis. Antisera were purified according to thecaprylic acid-saturated ammonium sulfate (SAS) method.[37] The responses of antibodies to the immunogen were evaluated using indirect ELISA.

Determination of antibody titer

An indirect ELISA was performed to detect the titer of antibody against BPA-OVA. To obtain the optimal concentrations of the coating antigen and antibody for the ELISA, a chequerboard method was applied as previously described.[33] Seven antibodies against BPA were optimized by criss-cross serial dilution analysis using BPA-OVA. Different concentrations of 50 μL of BPA-OVA were used to coat the microplate for 2 h at 37 °C. The wells of the microplate were washed three times with washing buffer (200 μL for each washing). The rest of the sites of the wells were blocked by adding 150 μL of blocking buffer for 1 h at 37 °C. 50

Table 1 Immunisation schemes and IC_{50} values for three BPA polyclonal antibodies

Antibody	Immunogen	Immunogen dose/mg	Scheme	Weeks between doses	IC_{50} for BPA/ng mL^{-1}
R001	BPA-HS1-BSA	0.1	A	2	50
R002	BPA-HS1-BSA	0.1	B	4	22
R003	BPA-HS1-BSA	0.05	C	8	5.51
R004	BPA-HS2-BSA	0.1	A	2	415
R005	BPA-HS2-BSA	0.1	B	4	62
R006	BPA-HS2-BSA	0.05	C	8	—
R007	BHPVA-BSA	0.1	B	4	100

μL of 2-fold serially diluted antibodies were incubated in the wells for 1 h at 37 °C after removing the blocking buffer. The plates were then washed again three times with washing buffer, and 50 μL of HRP-labelled goat anti-rabbit IgG diluted at a ratio of 1 : 5000 (v/v) was added to these plates followed by incubation at 37 °C for 45 minutes. Thereafter, the wells were washed four times with washing buffer and 50 μL of substrate was then added to each well. The plates were incubated at 37 °C for 20 minutes in the dark and the enzymatic reaction was stopped by adding 100 μL of 1 M H_2SO_4. The absorbance was measured at 450 nm using the Bio-Rad microplate reader.

Direct competitive ELISA procedure

Characterization and analysis of BPA using direct competitive ELISA was performed as previously described,[38] briefly; microtiter plates were coated with 50 μL of fixed concentration of antibody in coating buffer and incubated at 37 °C for 2 h. Then the plates were washed three times with 200 μL washing buffer. The direct competition step was carried out by adding 50 μL of various dilutions of standard solution and 50 μL of HRP conjugate (dilution 1 : 2000 v/v). After incubation at 37 °C for 45 minutes, the plates were washed four times using 200 μL washing buffer. 100 μL of substrate solution was added to each well followed by incubation at 37 °C for 20 minutes in the dark. 100 μL of H_2SO_4 was then added to stop the enzymatic reaction after incubation for 20 minutes at 37 °C in the dark. The absorbance was measured at 450 nm using the Bio-Rad microplate reader.

Indirect competitive ELISA procedure

According to a previously published report,[33] the indirect competitive ELISA for the characterization and analysis of BPA was performed as follows: microtiter plates were coated with 50 μL of BPA-OVA in coating buffer for 2 h at 37 °C. The wells were then washed three times with 200 μL washing buffer and the remaining sites were blocked using 150 μL blocking buffer for 1 h at 37 °C. The blocking buffer was removed and the competition step was carried out for 1 h at 37 °C by adding 50 μL of various dilutions of standard solution and 50 μL of a fixed concentration of antibody. After washing the plates three times, 50 μL of HRP- labled goat anti-rabbit IgG was added at a dilution of 1 : 5000 (v/v) followed by incubation at 37 °C for 45 minutes. After washing four times with washing buffer, 100 μL of substrate solution was added to each well. The plates were placed in the dark for 20 minutes at 37 °C for colour development, 100 μL of H_2SO_4 (1 M) was added to each plate to stop the enzymatic reaction. The absorbance was measured at 450 nm using the Bio-Rad microplate reader.

Assessment of antibody specificity

The capability of recognition of the antibody R003 to BPA, BHPVA, BPA-HS1, BPA-HS2, SAL and DES was investigated using the proposed direct competitive ELISA, and was determined by measuring their IC_{50} values in the direct ELISA. Cross-reactivity (CR) was calculated using the formula: CR (%) = (IC_{50} of BPA/IC_{50} of testing compound) × 100%.

Fortification experiment

Blank urine samples were used for the validation study using two competitive ELISAs. Inter-assay and intra-assay coefficient of variations (CVs) were determined by analysis of samples fortified with BPA at 1, 10, and 100 ng mL^{-1}. The recovery (%) of the fortified BPA was calculated and the repeatability of the method was established using fortified duplicate blanks at the levels 1, 10 and 100 ng mL^{-1}.

Results and discussion

Synthesis of hapten and BPA conjugates

BPA cannot initiate an immune response due to its low molecular weight. So in our present study, we attached BPA to BSA to induce an immune response. Due to the absence of a coupling group (such as NH_2, SH and COOH) in the structure of BPA, BPA haptens were synthesized to add the COOH group. The hapten's structure plays an important role in the production of sensitive antibodies. In the present study, three BPA haptens (BHPVA, BPA-HS1 and BPA-HS2) were designed for further conjugation. BHPVA has the COOH group and can be coupled to BSA directly without derivatization. As shown in Scheme 1, BPA-HS1 was synthesized following derivatization with ethyl bromoacetate, while BPA-HS2 was synthesized following derivatization with PABA. Two derivatives were identified by LC–electrospray MS. In the negative mode of MS, the theoretical molecular ion [M − H]− *m*/*z* of BPA-HS1 was 285 and a molecular peak *m*/*z* 285 was seen. The theoretical molecular ion [M − H]− *m*/*z* of BPA-HS2 was 375 and a molecular peak *m*/*z* 375 was seen.

Using the active ester method, the COOH groups on BPA-HS1, BPA-HS2 and BHPVA were conjugated with the NH_2 group on BSA to produce the immunogen, the COOH group on BPA-HS1 was conjugated with the NH_2 group on OVA to to produce the coating antigen and with HRP to produce the peroxidase tracer. Fig. 1 shows the UV absorption spectra of the immunogen and coating antigen. Almost the same specific UV absorption peaks at 278 nm are observed for BPA, BPA derivatives, BHPVA and proteins (BSA and OVA). On the basis of the wave shapes in the absorption spectra, it was quite difficult to identify the specific conjugate. Although, the concentrations of the free and conjugated proteins for UV scanning were the same but the peak height of each conjugated protein was significantly higher than that of the free proteins (Fig. 1) which indicated that successful conjugation of the haptens to the carrier proteins had occurred. Specific affinity of BPA-OVA to be recognized by the antibody was carried out. As show in Fig. 2, BPA-OVA had satisfactory binding capacity. The results indicated that the conjugation reaction did not affect the capacity of the BPA, which coupled to OVA, to be recognized by its specific antibody.

Characterization of the antibodies

Three BPA conjugates were used as immunogens and three schemes were used for immunization. Seven antibodies obtained from all the rabbits after several immunizations were monitored by indirect competitive ELISA. As shown in Table 1, R003 has the most sensitivity and could only exhibit an IC_{50}

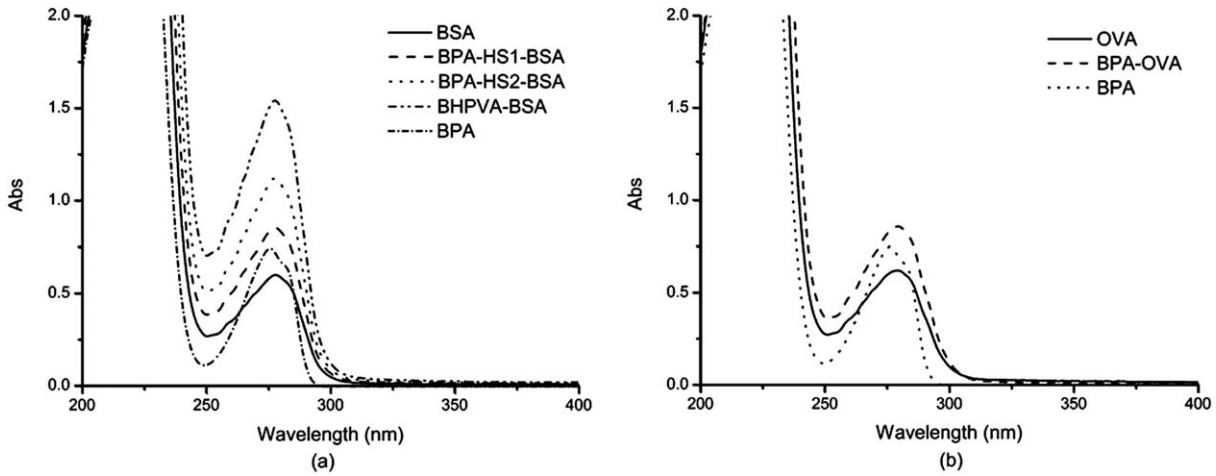


Fig. 1 (a) UV absorption spectra of BPA and immunogen. (b) UV absorption spectra of BPA and coating antigen.

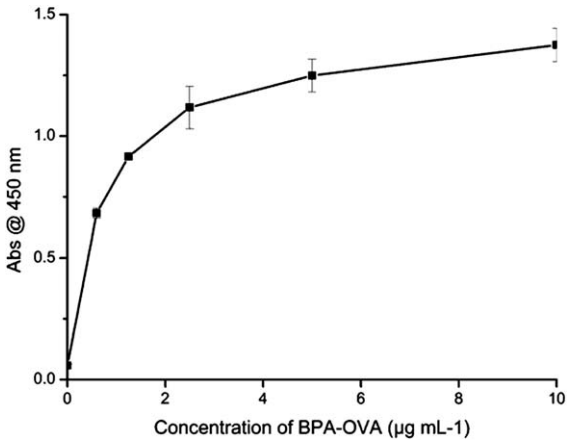


Fig. 2 Characterization of BPA-OVA. Various dilutions of BPA-OVA in the coating buffer were added to the wells of the microplate. Antibody R003 (1 : 100 000 v/v) and HRP-labled goat anti-rabbit IgG (1 : 5000 v/v) were used in the binding study.

value of 5.5 ng mL^{-1}. Through the analysis of different immunogens, we found that BPA-HS1-BSA could produce the most sensitive antibody compared with those produced by BPA-HS2-BSA and BHPVA-BSA. The production of highly sensitive antibodies is mainly influenced by several factors such as hapten structure, length of linker and position of conjugation in the hapten molecule. In our present study, the structure of BPA-HS2 contains PABA which acts as a linker for the conjugation of BPA and BSA. The presence of a phenyl structure in BPA-HS2 was found to be highly immunogenic and influenced the production of antibody against BPA. In the competitive ELISA, PABA was recognized by R004 and R005. The COOH group is naturally present in commercially available BHPVA without any derivatization but this COOH group is surrounded by two phenyl groupsas such the position of COOH is quite different from that of the phenyl groups in BPA and the antibody produced by BHPVA-BSA has low specificity to BPA. In the case of BPA-HS1, a simple linker was added on one side of BPA which increases the surface exposure of BPA in carrier protein by decreases the position change in space of BPA. Among all three, BPA-HS1 is the more appropriate for the production of highly specific antibodies against BPA. From the results of the immunizing schemes, we detected that the sensitivity of antibody towards BPA increased by decreasing the dose of immunogen and increasing the length of the rest periods between the booster immunizations. Individual differences among the animal groups may also influence the immune response but similar findings have also been noticed in our previous work (in the press) with COT immunization and our results are in agreement with a previously published report.[38] To make the conclusion more reliable and trustworthy, more studies with a large number of animals immunized using different immunization schemes may also need to be carried out.

Characterization of the ELISA

The titers of three antibodies (BPA-HS1, BPA-HS2 and BHPVA) were determined by checkerboard titration.[33] The optimal conditions for the ELISA were chosen when the absorbance value was about 1.0. In this experiment, the optimal concentration of the coating antigen was found to be 1 μg mL^{-1}. Under the optimal conditions, we found that the titers of the three antibodies of BPA-HS1, BPA-HS2 and BHPVA were 1 : 1 024 000, 1 : 16 000 and 1 : 64 000, respectively. As shown in Table 1, in the direct competitive ELISA format, the IC_{50} values of the antibodies produced by BPA-HS1-BSA, BPA-HS2-BSA and BHPVA-BSA were 5.5 ng mL^{-1}, 62 ng mL^{-1} and 100 ng mL^{-1} respectively. As the antibody produced by BPA-HS1 showed the highest sensitivity as compared to that of the other two antibodies, it was chosen to be characterized further and was used to detect the BPA levels in the urine samples.

In our previous study, we established an indirect competitive ELISA assay to quantify tartrazine in human urine samples.[33] However, the indirect competitive ELISA was reported to have a longer operation time and a higher LOD than the direct competitive ELISA.[39] To the best of our knowledge, our present study is the first simultaneous standardized two competitive (direct and indirect) ELISA for the detection of BPA in urine. We have also compared the characteristics of the two assays.

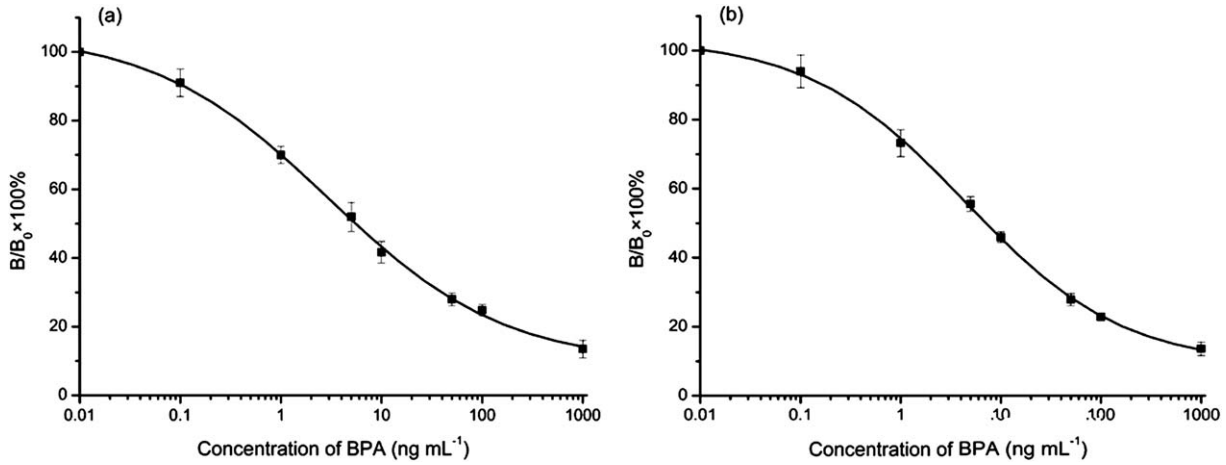
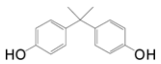
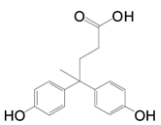
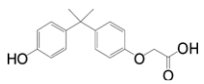
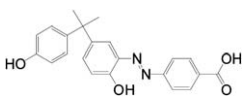
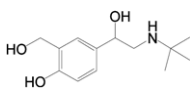
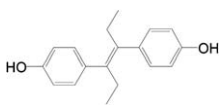


Fig. 3 (a) Standard curve for the direct competitive ELISA for the detection of BPA. Vertical bars indicate standard deviations of $B/B_0 \times 100\%$ ($n = 7$). (b) Standard curve of indirect competitive ELISA for detection of BPA. Vertical bars indicate standard deviations of $B/B_0 \times 100\%$ ($n = 7$).

Table 2 Cross reactivity of antibody R003 with BPA and other related compounds

Compound	Structure	IC_{50}/ng mL^{-1}	CR%
BPA		5.51	100%
BHPVA		>10 000	>0.05%
BPA-HS1		3.58	154%
BPA-HS2		156	3.35%
SAL		>10 000	>0.05%
DES		847	0.65%

The direct and indirect ELISA standard curves for BPA are presented in Fig. 3. The logit–log algorithm was used to establish the linear regression. The equations were $\ln(1 + B/B_0) = 4.46 - 0.6105 \log C_{BPA}$ ($R^2 = 0.9926$, $n = 7$) and $\ln(1 + B/B_0) = 4.54 - 0.6531 \log C_{BPA}$ ($R^2 = 0.9921$, $n = 7$). The CVs of the standards ranged from 7.9% to 14.4%. In the direct competitive ELISA, the antibody exhibited an IC_{50} value of 5.5 ng mL^{-1} and a LOD (calculated as +3SD for twenty replicates of zero calibrator) of 0.03 ng mL^{-1} whereas, in the indirect competitive ELISA, the antibody exhibited an IC_{50} value of 7.0 ng mL^{-1} and a LOD of 0.08 ng mL^{-1}.

The chemical compounds and their corresponding CR with the antibody are shown in Table 2. It can be seen that the strongest CR% response was obtained to BPA-HS1 whereas, a low CR% was found with BPA-HS2 and DES. The antibody did not show any CR with other related compounds. The data showed that the immunoassays for BPA are highly selective.

Fortification experiment

In order to assess the ability of the assay to accurately quantify BPA in urine samples, we employed the two proposed ELISAs (direct and indirect) in the analysis of human urine in a fortification experiment. Known amounts of standard BPA (1, 10, 100 ng mL^{-1}) were added to blank urine samples. The inter-assay and intra-assay CVs, and recoveries (%) for the direct and indirect competitive ELISAs are shown in Tables 3 and 4 respectively. In the direct competitive ELISA, the intra- and inter-assay recoveries for fortified urine ranged from 97.34% to 119.42% and from 102.63% to 118.37%, respectively. The intra- and inter-assay CVs were found to be from 9.85% to 15.46% and

Table 3 Results of imprecision and recovery for BPA determination in blank urine samples by direct competitive ELISA

	Intra-assay					Inter-assay				
QC/ng mL^{-1}	N	Mean	SD	CV (%)	Recovery (%)	N	Mean	SD	CV (%)	Recovery(%)
100	4	111.87	11.02	9.85	111.87	6	102.63	10.89	10.61	102.63
10	4	9.73	1.50	15.46	97.34	6	11.84	0.87	7.37	118.37
1	4	1.19	0.12	10.47	119.42	6	1.12	0.16	14.07	112.02

Table 4 Results of imprecision and recovery for BPA determination from blank urine samples by indirect competitive ELISA

	Intra-assay					Inter-assay				
QC/(ng mL^{-1})	*N*	Mean	SD	CV (%)	Recovery (%)	*N*	Mean	SD	CV (%)	Recovery (%)
100	4	102.07	8.96	8.78	102.07	6	113.12	10.18	9.00	113.12
10	4	11.38	0.86	7.59	113.81	6	10.10	0.63	6.23	100.99
1	4	1.05	0.15	14.38	105.27	6	1.10	0.16	14.58	110.03

from 7.37% to 14.07%, respectively. In the indirect competitive ELISA, the intra- and inter-assay recoveries for fortified urine ranged from 102.07% to 113.81% and from 100.99% to 113.12%, respectively. The intra- and inter-assay CVs were found to be from 7.59% to 14.38% and from 6.23% to 14.58%, respectively. These results demonstrate the good accuracy and precision of the assay. The established direct competitive ELISA was also performed to detect the urine concentrations of BPA of 23 healthy individuals. The samples contained BPA at concentrations ranging from 0.07 to 31.74 ng mL^{-1}. Exposure to BPA may produce adverse health effects. Higher urinary concentrations of BPA are associated with cardiovascular complications and diabetes.[40] Due to the limited availability of epidemiological data, it is a bit difficult to develop the direct association between potential health effects and human exposure to BPA.[13] In order to avoid the difficulty in detecting the minimum exposure of BPA, the number of samples should be increased to calculate significant data. Here in our present study, we have developed a direct competitive ELISA for the detection of BPA. It has high sensitivity and specificity. It can be applied for the reliable detection of BPA in human urine.

Conclusion

In our present study, we designed three haptens of BPA and their corresponding immunogens for the detection of BPA in a simplified and sensitive way. We investigated the influence of the hapten structure, length of linker and position of conjugation on the hapten molecule on the production of antibody against BPA. The hapten structure of BPA was found to have a great impact on the production of antibody specific against BPA. Based on the high sensitivity and specificity of antibody against BPA, we simultaneously developed direct and indirect competitive ELISAs, and then compared them with each other. Furthermore, we applied the direct competitive ELISA to detect BPA levels in urine samples. Moreover, the two developed methods were also applied to analyze human urine in a fortification experiment. From the results of fortification experiments, both the ELISAs showed specificity and sensitivity for BPA but the direct competitive ELISA was found to be a highly specific and sensitive detection method for BPA as compared to the indirect competitive ELISA.

Conflict of interest

The authors declare that they do not have any conflict of interest for this article.

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