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Development of a radioimmunoassay for the measurement of Bisphenol A in biological samples

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ABSTRACT

Bisphenol A (BPA) is widely used in the manufacturing of polycarbonate plastic food and drink packaging. Possessing a weak estrogenic activity, BPA is listed among a growing list of endocrine disrupting compounds.

In this study, a polyclonal anti-BPA antibody was obtained by immunization with BPA-monocarboxymethylether covalently linked to BSA. The antibody demonstrates negligible cross-reactivity with most analogous BPA phenolic structures, and no cross-reactivity with endogenous steroids. An extraction step with ethyl acetate minimized matrix effects and allowed the BPA measurement in plasma and other biological samples.

Recovery after loading test was 96 ± 4% and dilution tests had a linear profile ($r^2 > 0.93$). The limit of detection of the BPA RIA was 0.08 μg L^{-1} with an IC50 of 1.25 μg L^{-1}. The intra- and inter-assay coefficients of variation were 5.6 and 8.6%, respectively at a BPA concentration of 0.7 μg L^{-1} and 6.9 and 5.7% at a BPA concentration of 1.3 μg L^{-1}. A significant correlation was found between the values obtained by the RIA and HPLC–MS ($r^2 = 0.92$) or HPLC coupled to a fluorescence detector ($r^2 = 0.80$).

In conclusion, we described a BPA-RIA that is a suitable tool for evaluating human exposure to BPA.

1. Introduction

Bisphenol A (4,4′-dihydroxy-2,2-diphenylpropan; BPA) is a raw material used in the production of epoxy resins and polycarbonate plastics which are widely used in dentistry, food and drink packaging, and water pipes [1]. Human exposure to BPA exposure may occur through direct contact with BPA, or by consumption of food or drink that has been exposed to material containing BPA.

BPA binds to both the estrogen receptor ERα and ERβ [2], displaying a higher binding affinity for ERβ [3], and exerts either estrogenic or anti-estrogenic activity *in vitro* [4,5]. Studies in laboratory animals have been conducted, and, although the data have been largely controversial as a result of the high doses of BPA used in most experiments, they have suggested a potential link between BPA exposure and reproductive disorders, prostate and breast cancers, diabetes, and behavioral disorders. More recently, it has been shown that BPA, within the range found in human serum, could regulate pancreatic insulin synthesis in intact islets of Langerhans by a mechanism mediated by the estrogen receptor ERα, suggesting that BPA exposure might be involved in blood glucose homeostasis disorders [6]. In contrast, the Food and Drug Administration (FDA) has concluded that an adequate margin of safety exists for BPA at current levels of exposure from food contact. A recent study by Lang et al. [7] showed that serum concentrations of BPA are significantly correlated with markers of liver damage, such as increased serum glutamyltransferase levels, which were predictive of metabolic and cardiovascular disease [8].

The standard methods for BPA measurement use mass spectrometry (MS) coupled to gas chromatography (GC–MS, GC–MS–MS) or to liquid chromatography (LC–MS and LC–MS–MS) and liquid chromatography with different detections (fluorescence, electrochemical, UV) [9]. Although highly reliable, these techniques require extensive cleanup and sample treatment with derivatization steps. They are expensive and time-consuming, and require high technological instrumentation. These constraints have reduced the possibilities of exploring the implications of BPA expo-

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sure in humans. In contrast, immunochemical techniques using highly specific antibodies are generally sensitive enough for analysis from a small sample volume, and are adapted to automated systems. With a relatively low cost, they are useful for developing high-throughput screening methods. Several validated Enzyme-Linked Immunosorbent Assays (ELISA) measuring BPA have been reported [10,11], however, these ELISA methods display a poor correlation with the LC-ECD method, and the different ELISA kits correlate poorly with one another. Moreover, ELISA methods may over-estimate BPA in biologic samples due to low antibody specificity and biological matrix effects [12,13].

In epidemiological studies, BPA levels have been measured in human fluids by using different analytical techniques [7,14,15]. According to these studies, unconjugated BPA concentrations in human serum ranged from 0.2 to 20 ng mL^{-1}.

The aim of the current study was to develop a simple and cheap radioimmunoassay (RIA) which allows accurate measurement of unconjugated (free) BPA in a small volume of biological samples.

2. Materials and methods

2.1. Synthesis of the immunogen

BPA was derived to BPA-monocarboxymethylether (BPA-CME), which was then covalently linked through peptidic bonds to lysine residues of bovine serum albumin (BSA). BPA (2 g, 8.77 mmol) dissolved in a mixture of ethanol (36 mL) and tetrahydrofuran (THF) (10 mL), was treated with a slight excess of sodium (0.25 g, 10.86 mmol) and monoalkylated with a half-stoichiometric amount of sodium chloroacetate (0.51 g, 4.39 mmol). After 48 h under reflux, the reaction mixture was purified by preparative thin layer chromatography (TLC) using chloroform–acetone–acetic acid (70/20/10) as mobile phase. The identity was confirmed by nuclear magnetic resonance (NMR).

The carboxylic group of BPA-CME was covalently coupled to the epsilon-amino group of lysine side-chains of the carrier protein BSA through amide bonds using the active *N*-hydroxysuccinimide ester method in the presence of 1.3-dicyclohexyl carbodiimide (DCI). In brief, BPA-CME (20 mg, 100 μmol) was dissolved in THF (4 mL) to which was added *N*-hydroxysuccinimide (NHS) (12.4 mg, 110 μmol) and DCI (20.7 mg, 100 μmol). The reaction mixture was stirred at room temperature for 2 h. TLC on silica gel developed in ethyl acetate indicated the appearance of the ester. The reaction mixture was centrifuged, and the crude supernatant was used for conjugation with BSA. A solution of 102 mg of BSA in 10 mL of 0.1 M sodium bicarbonate, to which was added 4.5 mL of THF, was continuous stirred while the solution of NHS ester was added dropwise. The mixture was then stirred for 2 h at room temperature. The hapten-BSA conjugate was dialyzed against 0.1 M $NaHCO_3$, and subsequently purified on a Sepharose CL4B column. The hapten/carrier protein ratio (6/1) of the conjugate was estimated by ultraviolet spectrometry.

2.2. Production of antisera

Four white New-Zealand rabbits were immunized by subcutaneous dorsal injection of immunogen (250 μg per animal) in complete Freund's adjuvant. Booster injections were given by the same route at monthly intervals. Blood samples were tested for the level of anti-BPA antibodies 1 week after each injection.

2.3. Synthesis of ^{125}I-labeled BPA

BPA was directly iodinated according to the method of Hunter and Greenwood [16]. In brief, 1 mg mL^{-1} BPA in methanol was first diluted 10-fold in 50 mmol L^{-1} sodium phosphate pH 7.4. A 10 μL aliquot of this solution corresponding to 1 μg of BPA was added to a reaction vial containing 7.4 MBq of carrier-free [^{125}I] NaI (from Amersham International, volumic activity 3.7 GBq mL^{-1}). Then, chloramine T (10 μL of a 1 mg mL^{-1} sodium phosphate solution) was added to the iodination mixture and allowed to react for 20 s. The reaction was then stopped by the addition of 50 μL of sodium metabisulfite (4 mg mL^{-1}). The tracer was purified by HPLC. Separation was achieved with a 250 × 4.6 analytical column (C18 Nucleosil, Macherey Nagel) with 50% methanol as mobile phase. Fractions were collected every minute and counted in a gamma-counter. The fractions containing the main radioactive peak were pooled and stored at −20 °C. This radioactive HPLC peak was found to be superimposable to that of a radioinert mono-2-iodo-BPA reference sample.

2.4. RIA procedure

BPA was dissolved in methanol to produce a stock solution of 1 μg mL^{-1}. For each assay, the stock solution was diluted in physiological serum to provide standards over a range of 0.08–5 μg L^{-1}.

BPA was measured after an extraction step as follows: standard or plasma samples (0.25 mL) were pipetted into glass tubes containing 0.75 mL of NH_4OH 5% solution. After addition of 2.5 mL ethyl acetate (quality Pestipur) and agitation on a Vortex, 2 mL of upper organic phase was transferred into a glass tube and evaporated to dryness under a nitrogen stream. The dry residue was dissolved in 0.5 mL of assay buffer (1.78 g $Na_2HPO_4{\cdot}2H_2O$, 0.31 g $NaH_2PO_4{\cdot}2H_2O$, 7.36 g NaCl, and 1 g BSA per liter).

BPA concentration was measured in duplicate by RIA as follows: 100 μL of ^{125}I-BPA tracer (∼20 000 cpm) was mixed with the standard or the sample (200 μL) and 100 μL of antibody solution, diluted 5000-fold in assay buffer. The final volume of 500 μL was completed for each sample with assay buffer. Samples were incubated for 18 h at 4 °C. Bound and free BPA were separated by addition of 500 μL charcoal suspension (0.27 g L^{-1} Dextran T70 and 2.7 g L^{-1} charcoal Norit-A in assay buffer). After 15 min incubation at 4 °C and centrifugation (3000 × *g*) for 15 min at 4 °C, the radioactivity of the supernatant, containing the BPA bound to antibody, was counted in a gamma-counter (Cobra, Packard).

2.5. Analytical performance

The specificity of the RIA was evaluated by determining the cross-reactivity of the antibodies with several structures that are analogous to BPA: 4,4′-ethylidene diphenol, 2-phenylphenol, 4-tert-octylphenol, 4-nonylphenol, 4-cumylphenol, bis(4-hydroxyphenyl)methane (Bisphenol F), 17β estradiol, and diethylstilbestrol. The detection limit was defined as the concentration giving a displacement of 3 standard deviations (SD) of maximum binding counts (n = 10).

To determine the linearity of response to sample dilution, we carried out 4 dilutions from plasma with a high concentration of BPA (15 μg L^{-1}). The measured BPA concentrations were compared with expected values.

To establish inter-assay precision, six replicated standard curves were set up using 2 plasma control preparations containing 0.7 and 1.3 μg L^{-1} of BPA. To ascertain the intra-assay precision, the plasma controls were also assayed in 6 replicates, in one single run.

The recovery tests were carried out by addition of 0.25, 0.5 and 1.5 μg L^{-1} of BPA to human plasma sample replicated 6 times.

The reliability of our method was tested in two steps. Firstly, BPA values from extracted samples from spiked water (n = 20)