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Sample clean-up with sol–gel enzyme and immunoaffinity columns for the determination of bisphenol A in human urine

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Abstract

The paper describes the development of a simple and highly selective analytical method for the determination of free and total bisphenol A in urine samples. Free bisphenol A levels can be determined after sample clean-up using sol–gel immunoaffinity columns containing anti-bisphenol A antibodies. In determining total bisphenol A levels, the sample pre-treatment procedure consists of sample preparation using an on-line combination of two sol–gel columns, an enzyme column containing glucuronidase and arylsulfatase, and an immunoaffinity column. Bisphenol A can then be quantified by high-performance liquid chromatography and fluorescence detection. The mean recovery was found to be 78% with a standard deviation of 3.4%, the LOD (S/N = 3) was 0.2 ng/ml. The method was applied to determine free and total urinary BPA levels of healthy adults and dialysis patients.

Keywords: Bisphenol A; Sol–gel; Enzyme column; Immunoaffinity column; Urine; Dialysis patients

1. Introduction

Humans are exposed to a number of substances – so-called endocrine-disrupting compounds (EDCs) – which have the potential to disturb the function of the hormonal system. The estrogenic activity of 2,2-bis(4-hydroxyphenyl)propane, more commonly known as bisphenol A (BPA), has been reported for the first time in 1993 [1]. Meanwhile endocrine effects of BPA have been investigated in numerous *in vitro* and *in vivo* studies, e.g. cell proliferation assays using MCF-7 human breast cancer cells [2,3], recombinant yeast cell assays [4,5] and the rodent uterotrophic response assay [6].

BPA is mainly used to produce polycarbonates which in turn are converted to various consumer goods including food contact containers, compact discs and medical devices. In addition, BPA is used to manufacture epoxy resins which are applied as linings in food and beverage cans. Several studies have already indicated leakage of BPA traces from polycarbonate containers [7,8] and epoxy linings in food [9–12].

When incorporated in humans, BPA is rapidly metabolised in the liver, mainly to BPA glucuronide, and excreted in urine [13]. Conjugation of BPA has been reported to have a considerable effect on lowering its estrogenic activity [14].

Exposure of humans to BPA can be assessed by determining BPA concentrations in biological fluids like blood or urine. However, in spite of the use of selective separation and sensitive detection methods, it is not possible to determine these low levels without carrying out selective sample pre-treatment steps. Till now, solid phase extraction (SPE) is the most frequently applied clean-up method [13,15–21]. Recently, Kawaguchi et al. developed a method based on stir bar sorptive extraction to isolate BPA from human urine samples [22]. Zhao et al. investigated the applicability of immunoaffinity columns for the clean-up of serum samples [23]. Total BPA levels including free and conjugated BPA are usually determined after an additional pre-treatment step carried out to cleave the BPA conjugates. Deconjugation is usually achieved by adding the enzyme glucuronidase or a mixture of glucuronidase and sulfatase to the sample solution and incubating at 37 °C for 1–3 h [13,17–22,24–26]. BPA can be quantified either by GC–MS [13,18–20,22] or HPLC with fluorescence [23–25], electrochemical [15–17,27] or mass spectrometric detection [13,15,21,26].

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Our group has recently developed analytical methods to determine BPA in various food samples [12,28–30]. Interfering matrix components were selectively removed with immunoaffinity columns prepared by entrapping polyclonal anti-BPA antibodies in porous sol–gel glass. The present study aimed at investigating the applicability of sol–gel immunoaffinity columns for clean-up of urine samples in the determination of low BPA concentrations. Enzymatic cleavage of urinary conjugates in the most common way – by adding the enzyme to the sample solution – suffers from several disadvantages compared to using the enzymes in immobilised form. Rather long incubation periods are needed and at the end of the incubation time an extraction or centrifugation step is required to remove the enzymes from the sample solution. One of our recent studies indicated a high potential of sol–gel columns containing immobilised glucuronidase and arylsulfatase for cleaving urinary conjugates [31]. In the present study, we investigated the applicability of sol–gel enzyme columns to hydrolyse BPA urinary conjugates. The sample pre-treatment method developed includes both sol–gel enzyme and immunoaffinity columns and was applied to determine BPA concentrations in human urine samples. In a recently published paper Shintani had reported BPA migration from artificial dialysers [16]. In order to get a first idea on the possible BPA exposition of dialysis patients, we compared urinary BPA levels from healthy adults and dialysis patients.

2. Experimental

2.1. Materials and reagents

Purified polyclonal anti-bisphenol A (BPA) antibodies (5 mg/240 μl phosphate-buffered saline (PBS)) were a gift from Japan EnviroChemicals (Tokyo, Japan). Bisphenol A (BPA) and ethyl acetate were obtained from Sigma (St. Louis, MO, USA). Bisphenol A mono-β-(D)-glucuronide was kindly delivered from Dow Chemical Company (Michigan, USA). *Helix pomatia* β-glucuronidase (EC 3.2.1.31)/arylsulfatase (EC 3.1.6.1) was obtained from Roche. Acetonitrile (ACN), HPLC gradient grade, was purchased from Fisher Scientific (Leicestershire, UK). Tetramethoxysilane (TMOS) was from Fluka (Buchs, Switzerland).

Three millilitre SPE-C_{18} columns packed with 500 mg Isolute were from International Sorbent Technology (Mid Glamorgan, UK). Creatinine was determined using the Creatinine Jaffe Kinetic Fluid test from Centronic (Notzing, Germany).

2.2. Standard solutions and buffers

BPA stock solutions were prepared by dissolving 10.0 mg of BPA in 100 ml of ACN. Working standard solutions were prepared by diluting the stock solutions with water. Phosphate-buffered saline, pH 7.6, was made by dissolving 21.25 g NaCl, 3.9 g Na_2HPO_4 and 1.15 g NaH_2PO_4 in 2.5 l of H_2O. The acetate buffer used was a 0.1 M sodium acetate buffer pH 5.2 containing 0.5 M sodium chloride. Bidistilled water was used in all experiments.

2.3. Instrumentation

Immunoaffinity and enzyme columns were operated using a peristaltic pump (Econo Pump, Model EP-1, BioRad, Hercules CA, USA).

In the present study, two HPLC systems were used. HPLC system 1 was used to develop the analytical method and to determine BPA concentrations in urine samples, whereas HPLC system 2 was used to verify the identity of BPA.

HPLC system 1 consisted of a Merck-Hitachi L6200 Intelligent HPLC pump, a column thermostat (Merck-Hitachi 655A-52) and a six-port injection valve (Rheodyne) equipped with a 100 μl injection loop. Detection of BPA was carried out with a fluorescence detector (Merck-Hitachi F-1080) at 275/305 nm. Chromatographic peaks were integrated using the Stratos version 3.0 software (Polymer Laboratories, Darmstadt, Germany).

HPLC system 2 consisted of a Hewlett Packard Series 1100 gradient pump (Agilent, Vienna, Austria) and a HP Series 1100 autosampler. A HCT plus ESI-Ion trap mass spectrometer (Bruker Daltonics, Vienna, Austria) was used for detection in the negative mode. The selected temperature for the heated capillary was 300 °C. The dry-gas flow was set at 10 l/min. The $[M\text{-}H]^-$ of BPA, 227.2 *m/z*, was isolated and fragmented. The scan area was adjusted from 120 to 235 *m/z*.

2.4. Preparation of sol–gel columns

2.4.1. Immunoaffinity columns

Immunoaffinity columns were prepared by entrapping 1 mg of anti-BPA antibodies in sol–gel glass as described previously [28]. After usage, the columns were regenerated with 20 ml of PBS and stored at 4 °C.

2.4.2. Enzyme columns

The crude enzyme preparation containing β-glucuronidase and arylsulfatase was dialysed against PBS using a molecular porous membrane with a molecular weight cut-off of 12–14 kDa (Medicell, London, UK). After dialysis, the protein concentration was determined by the Bradford assay [32]. Co-entrapment of β-glucuronidase and arylsulfatase in sol–gel glass was carried out according to a previously described procedure [31]. In the present study, 315 μl of the dialysed enzyme solution (43 mg protein/ml) and 1685 μl of PBS were mixed with 2 ml of pre-hydrolysed TMOS. At a weight loss of 50%, the sol–gel glass (2 g) was manually crushed and packed into a 8 ml glass column (Merck) equipped with a glass microfibre filter GF/F from Whatman (Kent, UK). The columns were flushed sequentially with 20 ml of PBS and 20 ml of acetate buffer and stored at 4 °C. Before usage the columns were pre-conditioned with 20 ml of PBS, after usage they were regenerated with 20 ml of acetate buffer.

2.4.3. Enzyme/antibody column type A

In a pre-weighed beaker, 315 μl of the dialysed enzyme solution (43 mg protein/ml), 100 μl of the anti-BPA antibody solution (1 mg anti-BPA antibody) and 1585 μl of PBS were