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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

mixed with 2 ml of pre-hydrolysed TMOS. The resulting silicate glass (2 g) was packed into a 8 ml glass column (Merck) equipped with a glass microfibre filter, washed with 20 ml of PBS and stored at 4 °C. After usage the columns were regenerated with 20 ml of PBS.

2.4.4. Enzyme/antibody column type B

Sol–gel glasses containing either antibodies or the enzymes glucuronidase and sulfatase were prepared as described above. The enzyme/antibody column type B was prepared by packing into a 8 ml glass column 1 g of sol–gel glass containing anti-BPA antibodies, covering the glass material by a glass fibre filter and adding a second layer of sol–gel glass (2 g) containing glucuronidase and arylsulfatase.

2.5. Sample preparation

2.5.1. Immunoaffinity chromatography

After mixing 10 ml of urine with 1.5 ml PBS and adjusting the pH to 7.2–7.4, the sample was applied to the immunoaffinity column at a flow-rate of 1 ml/min. After washing the column with 6 ml of ACN-water (5:95, v/v), BPA was eluted with 4 ml of ACN-water (40:60, v/v). The volume of eluate was reduced to <1 ml under a stream of nitrogen, transferred into a 1 ml measuring flask and filled up to the ring mark with water.

2.5.2. Enzymatic cleavage in sol–gel columns

The enzyme column was conditioned with 20 ml of PBS. After mixing 10 ml of urine with 1.5 ml PBS and adjusting the pH to 7.2–7.4, the sample was centrifuged at 700 × *g* for 2 min. After applying the supernatant, the enzyme column was flushed with 10 ml of PBS.

2.5.3. Enzymatic cleavage in solution (reference method)

Ten millilitres of urine were mixed with 1.5 ml acetate buffer and 100 μl dialysed enzyme solution (43 mg protein/ml). After incubation under gently stirring at 37 °C overnight the pH was adjusted to 7.6 with diluted NaOH. The sample was centrifuged at 700 × g for 2 min.

2.5.4. Liquid/liquid extraction

After diluting 10 ml of the urine sample with 2 ml of 2 M HCl, 32 ml of ethyl acetate were added. The mixture was vortexed for 1 min and centrifuged at 700 × g for 2 min. After evaporating the ethyl acetate phase under a stream of nitrogen the residue was dissolved in 5 ml of PBS.

2.5.5. Solid phase extraction

Ten millilitres of the urine sample were applied to a SPE-C_{18} column pre-flushed with 6 ml of ACN and 6 ml ACN-water (10:90, v/v). After washing the column with 5 ml ACN-water (20:80, v/v) elution was carried out with 4 ml of ACN into a 5 ml flask. The volume of the eluate was reduced to <1 ml under a stream of nitrogen and filled up with water.

2.5.6. Stability of BPA glucuronide

After spiking a blank urine sample with either about 500, 750 or 1000 ng of BPA glucuronide the urine sample was applied to a sol–gel column containing 2 g of pure sol–gel glass. After washing the column with 6 ml of ACN-water (5:95, v/v), elution was carried out with 4 ml of ACN-water (40:60, v/v). The eluate was collected in a 5 ml flask and filled up with water.

2.5.7. Blank urine sample

A blank urine sample – free from both BPA and BPA conjugates – was prepared by mixing 10 ml of a urine sample with 1.5 ml acetate buffer and 100 μl dialysed enzyme solution (43 mg protein/ml). After incubation at 37 °C overnight the pH was adjusted to 7.6 with diluted NaOH. After centrifugation (700 × *g*, 2 min) the supernatant was applied to the immunoaffinity column. Since BPA was retained in the column, blank urine free from BPA could be collected at the end of the column.

2.6. HPLC separation and detection

2.6.1. Phase systems

In HPLC system 1, the analytical column was a LiChroCART RP-100-18, 250 mm × 4 mm i.d., 4 μm (Merck) operated with a mobile phase consisting of ACN-water (30:70, v/v). All separations were carried out at 25 °C applying a flow-rate of 1 ml/min. The injection volume was 100 μl.

In HPLC system 2, a RP-18 column, 150 mm × 2.1 mm i.d., 3 μm (ACE, Aberdeen, Scotland) was used. Elution of BPA was carried out with ACN-water (47:53, v/v) applying a flow-rate of 0.2 ml/min. The injection volume was 30 μl.

2.6.2. External calibration

HPLC system 1 was calibrated by injecting seven standard solutions in the concentration range from 0.5 to 100 ng BPA/ml water.

2.7. Analysis of urine samples

Urine samples were collected at different times throughout the day. Urine samples from uremia patients were kindly provided from the Division of Nephrology and Dialysis, Department of Medicine III, Medical University of Vienna, Vienna, Austria. The samples were stored in plastic urine collecting beakers at −18 °C. Urine samples from healthy adults were collected in glass beakers and stored at −18 °C.

2.7.1. Leakage of BPA from urine collecting beakers

Ten millilitres of a urine sample were stored in a plastic beaker for 10 days at −18 °C. At the end of the storage time, the concentration of free BPA was determined. For comparison, an aliquot of the same urine sample was analysed immediately after urine collection.

2.7.2. Determination of free BPA

After mixing 10 ml of urine with 1.5 ml PBS and adjusting the pH to 7.2–7.4, the sample was directly applied to the immunoaffinity column at a flow-rate of 1 ml/min. The

immunoaffinity column was operated as described in Section 2.5.1.

2.7.3. Determination of total (free plus conjugated) BPA

The enzyme column was conditioned with 20 ml of PBS. After mixing 10 ml of urine with 1.5 ml PBS and adjusting the pH to 7.2–7.4, the sample was centrifuged at 700 × *g* for 2 min. The supernatant was applied to the enzyme column being on-line coupled to the immunoaffinity column (see Fig. 1B). After flushing the columns with 10 ml of PBS, the enzyme column was removed. After washing the immunoaffinity column with 6 ml of ACN-water (5:95, v/v) BPA was eluted with 4 ml ACN-water (40:60, v/v) into a 5 ml flask which was filled up to the ring mark with water.

2.7.4. Standard addition method

Since the purity of BPA glucuronide was not known, urine samples were spiked with BPA and not with BPA glucuronide. One unspiked and three spiked samples (from 1 to 4 ng BPA/ml sample) were analysed as described in Section 2.7.3. Peak areas were plotted against the amount of analyte added. The recovery was determined by dividing the slope of the linear regression line for the standard addition by the slope of the linear regression line of standard solutions.

3. Results

3.1. Immunoaffinity chromatography

The characteristics of sol–gel immunoaffinity columns containing anti-BPA antibodies, e.g. retention mechanism and binding capacity, as well as the optimal sample feeding and elution conditions have been published in a previous paper describing the development and application of an analytical method for the determination of BPA in food samples [28]. In the present study, preliminary experiments were carried out to investigate if the operation conditions optimised for the clean-up of food samples had to be adapted for the pre-treatment of urine samples. Before urine samples were loaded to the immunoaffinity column, pH was adjusted to about 7.2–7.4 to enable retention of BPA due to interactions with the immobilised antibodies. In the first experiment, the immunoaffinity columns were washed with 5 ml of PBS before BPA was eluted with ACN-water (40:60, v/v). In these cases a matrix peak was overlapping with the BPA peak. However, by washing the immunoaffinity column with 6 ml of ACN-water (5:95, v/v) the interfering matrix component could be completely removed. Due to the higher efficiency in removing matrix compounds, in further experiments immunoaffinity columns were washed with 6 ml of ACN-water (5:95, v/v).

3.2. Enzymatic cleavage

In one of our previous papers, we have shown that sol–gel columns containing glucuronidase and arylsulfatase could be used to cleave urinary conjugates within an incubation time of just a few minutes [31]. In addition, the columns proved to be very stable and could be used for a high number of cleavage cycles. In the present study, it was investigated if the sol–gel enzyme columns could also be used to cleave BPA conjugates, e.g. BPA glucuronide and BPA sulfate. We investigated four different approaches to solve this problem (see Fig. 1).

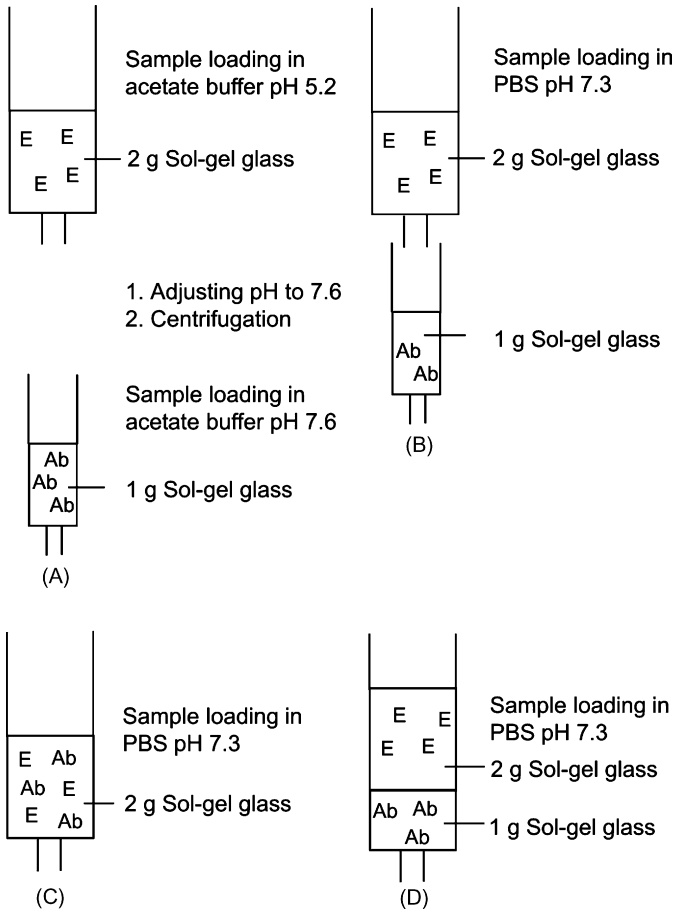

Fig. 1. Sample clean-up operation principles. (A) off-line combination of an enzyme column with an antibody column, operated with two different mobile phases, (B) on-line combination of an enzyme column and an antibody column both operated with the same mobile phase, (C) a single column packed with a sol–gel column bed with both the two enzymes and the antibodies entrapped in the same pores (enzyme/antibody column type A) and (D) a single column with a bed consisting of two packing materials – the initial part containing the entrapped enzyme mixture followed by a part with entrapped antibodies (enzyme/antibody column type B). E: Enzyme, Ab: antibody.

(A) An off-line combination of an enzyme column with an antibody column, operated with two different mobile phases optimised for enzymatic cleavage or BPA retention by antibodies (see Fig. 1A)

After mixing 10 ml aliquots of three urine samples with 1.5 ml acetate buffer and adjusting the pH to 5.2, the samples were loaded to the enzyme column without additional pressure (drop rate about 0.5 ml/min). Deconjugated BPA was eluted by flushing the column with 10 ml of acetate buffer. In order to enable retention of BPA in the immunoaffinity column the pH of the eluate was adjusted to 7.6. Since at this pH a precipitate was formed, the solution had to be centrifuged before being loaded to the

Table 1
Comparison of total urinary BPA levels obtained after cleaving BPA conjugates in the enzyme column with those obtained by adding glucuronidase and arylsulfatase to the sample solution

Sample number	BPA concentration (ng/ml)	
	Enzyme column	Enzyme in solution
1	1.7	1.8
2	0.7	0.7
3	1.2	1.2

The values are arithmetic means calculated from two determinations.

immunoaffinity column in order to avoid column clogging. Washing and elution of the immunoaffinity column were carried out as described in Section 2.5.1. BPA levels determined by injecting aliquots of the eluates into HPLC system 1 are given in Table 1. In order to assess the cleavage efficiency of the sol–gel enzyme columns, the same three samples were also analysed by adding the enzymes glucuronidase and arylsulfatase to the sample solutions (see Section 2.5.3). The data in Table 1 shows that cleavage in the enzyme column within a few min is as efficient as incubation with the enzymes in solution overnight.

(B) An on-line combination of an enzyme column and an antibody column both operated with the same mobile phase (see Fig. 1B)

In the experiments described above, the enzyme and the immunoaffinity column were operated at different pH values – cleavage of BPA conjugates was carried out in acetate buffer pH 5.2, whereas pH 7.6 was applied to achieve retention of BPA in the immunoaffinity column. In order to simplify the sample pre-treatment procedure we looked for operation conditions applicable for both hydrolysis of BPA conjugates in the enzyme column and binding of BPA to the entrapped antibodies.

When acetate buffer pH 5.2 was used for loading BPA (5 ml of a 5 ng/ml solution) to the immunoaffinity column only 51% of the loaded amount could be recovered in the eluate. Increasing the pH of the feeding medium to 6.3 resulted in only a slightly higher recovery of 58%, indicating that the interactions between BPA and the entrapped antibodies are inhibited by acidic sample loading conditions. In the next experiment a urine sample was loaded onto the enzyme column in PBS pH 7.3. BPA was eluted by flushing the enzyme column with 10 ml of PBS pH 7.3 directly onto the immunoaffinity column (see Fig. 1B). After separating the two columns the immunoaffinity column was operated as described in Section 2.5.1. This operating procedure yielded the same BPA levels as the reference procedure consisting of enzymatic cleavage in solution followed by immunoaffinity chromatography carried out by loading the sample solution in PBS pH 7.6. These results indicate that PBS pH 7.3 is suitable for achieving high cleavage rates in the enzyme column and retention of BPA in the immunoaffinity column.

(C) Use of a single column (enzyme/antibody column type A) packed with a sol–gel column bed with both the two enzymes and the antibodies entrapped in the same pores (Fig. 1C)

It was investigated if one sol–gel column containing glucuronidase, sulfatase and anti-BPA antibodies can be used for both cleavage of BPA conjugates and enrichment of BPA in one step. The enzyme/antibody column type A (see Fig. 1C) was prepared as described in Section 2.4. After loading an urine sample, the column was washed with 10 ml of ACN-water (5:95, v/v). Since this type of column was packed with the double amount of sol–gel glass compared to the immunoaffinity column (2 g instead of 1 g), the elution volume was increased to 8 ml. However, in spite of the larger elution volume the BPA level found was only 12% of the level obtained by carrying out the procedure shown in Fig. 1A. The low recovery may be caused either by the formation of aggregates between enzymes and antibodies and/or slow kinetic.

(D) Use of a single column (enzyme/antibody column type B) with a bed consisting of two packing materials – the initial part containing the entrapped enzyme mixture followed by a part with entrapped antibodies (Fig. 1D)

In order to investigate the potential of dual bed columns, an enzyme/antibody column type B was prepared as described in Section 2.4 and tested with a BPA standard solution in scouting experiments. The column was packed with two layers of sol–gel glasses separated from each other by a glass fibre filter: glucuronidase and arylsulfatase were entrapped in the upper layer whereas the anti-BPA antibodies were immobilised in the lower sol–gel glass layer. After loading a BPA standard solution (750 μl of a 100 ng/ml solution in PBS pH 7.3) the column was washed with 15 ml of ACN-water (5:95, v/v). Since the composite bed volume was increased to 3 g of sol–gel glass, BPA was eluted with 15 ml of ACN-water (40:60, v/v). However, in spite of the large elution volume the recovery of BPA was only 66%. Since a low limit of detection can only be achieved when the analyte is eluted in a small elution volume, further experiments with this column type were not carried out.

All the following experiments were therefore carried out using the column system shown in Fig. 1B.

3.2.1. Selectivity of the analytical method

In order to demonstrate the selectivity gained by including the immunoaffinity column in the clean-up procedure, several urine samples were analysed by replacing immunoaffinity chromatography either by liquid–liquid extraction or solid phase extraction. Fig. 2 shows representative HPLC chromatograms of a BPA standard solution (10 ng/ml) (Fig. 2A) and of a urine sample obtained after cleavage in the enzyme column followed by clean-up with either liquid–liquid extraction (LLE) (Fig. 2B), SPE (Fig. 2C) or immunoaffinity chromatography (Fig. 2D). As can be seen, only immunoaffinity chromatography resulted in HPLC chromatograms free from interfering matrix compounds.

3.2.2. Verification of the identity of BPA

In order to confirm the identity of BPA the aliquot of a pretreated urine sample was analysed by LC–MS (HPLC system

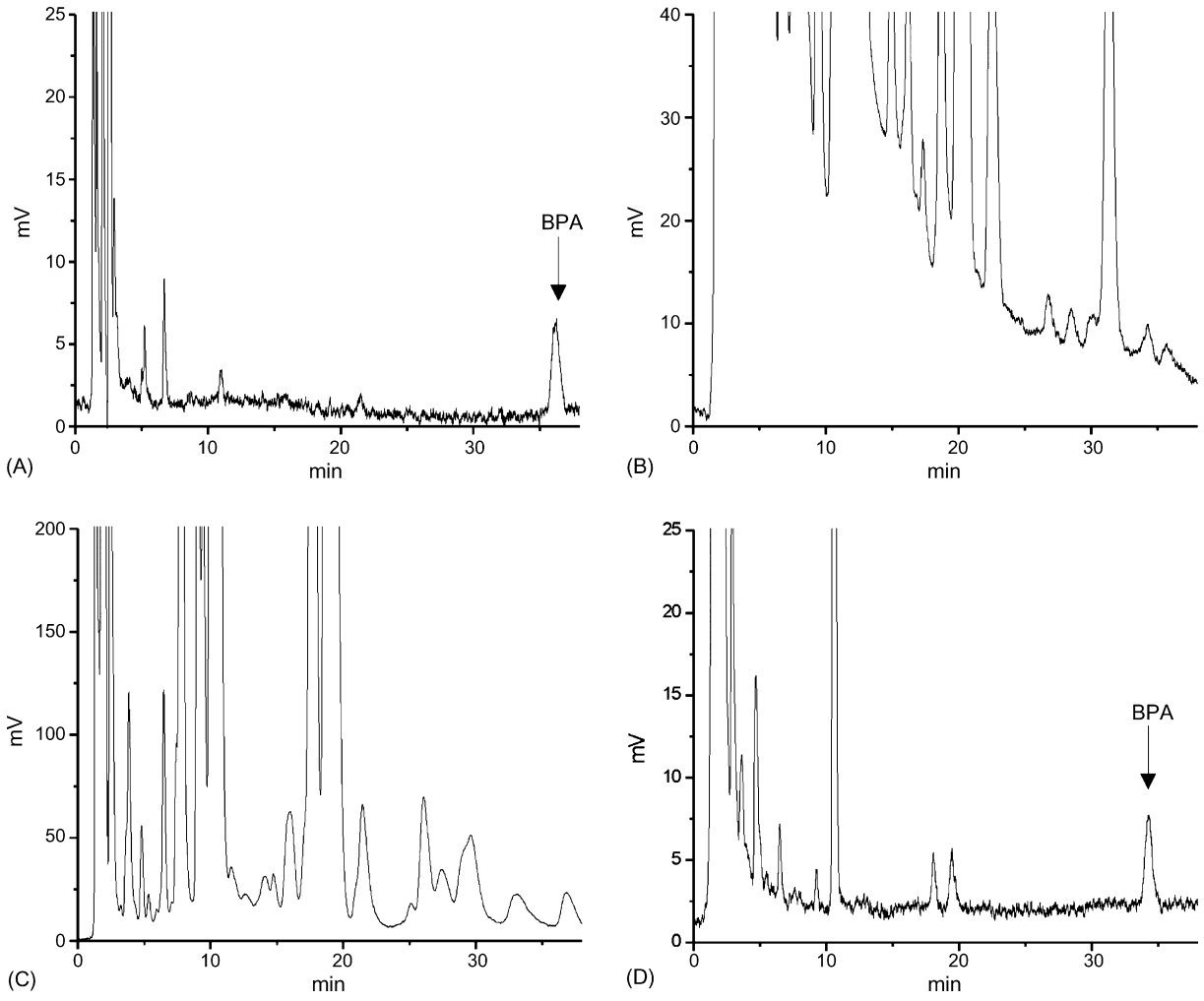

Fig. 2. Comparison of the selectivity of different sample clean-up methods. HPLC chromatograms obtained after injecting into HPLC system 1 (A) a BPA standard solution (10 ng/ml) and (B–D) a urine sample after cleavage in the enzyme column followed by clean-up with either (B) liquid–liquid extraction, (C) SPE or (D) immunoaffinity chromatography.

2). BPA was eluted in 5.5 min. The full scan MS spectrum showed a main signal at *m/z* 227 ion, which was assigned the $[M\text{-}H]^-$ ion. MS/MS analysis showed the most abundant fragment at *m/z* 212 which can be attributed to the cleavage of a C–C bond with a loss of a methyl group from the $[M\text{-}H]^-$ ion (see Fig. 3). The fragment ion at *m/z* 133 is assigned to a loss of a phenol group together with a hydrogen atom transferred from one of the methyl groups with formation of a double bond.

3.2.3. Stability of BPA glucuronide

Preliminary experiments have shown that BPA glucuronide can be stored at 4 °C for at least 3 days without being degraded to free BPA. However, at room temperature BPA glucuronide was completely cleaved to BPA within a few hours. Due to the instability of BPA glucuronide at room temperature we had to investigate if the applied sample pre-treatment conditions can cause undesired deconjugation of BPA conjugates which would introduce a systematic error leading to an overestimation of the actual free BPA concentrations. Since it was likely that – if at all – only a small percentage of BPA glucuronide is hydrolysed, the experiments had to be carried out with a large amount of BPA glucuronide to ensure that the mass of BPA formed surpassed the limit of detection. For these experiments, the enzyme column was replaced by a column packed with 2 g of pure sol–gel glass. After loading 10 ml of a standard solution containing about 100 ng BPA glucuronide/ml to the column, BPA was eluted with 10 ml of PBS buffer pH 7.3 directly onto the immunoaffinity column. The chromatograms obtained by injecting aliquots of the eluates into HPLC system 1 did not show a BPA peak, indicating that the applied clean-up procedure did not cause degradation of BPA glucuronide. The same experiment was also carried out with a blank urine sample (free from both BPA and BPA conjugates) spiked with BPA glucuronide (either 500, 700 or 1000 ng). In order to prepare blank urine, a urine sample was subjected to enzymatic cleavage and then loaded to the immunoaffinity column. Since BPA was retained in the column due to interactions with the anti-BPA antibodies, blank urine free from BPA could be collected at the end of the column. In the case of clean-up of the blank urine sample spiked with BPA glucuronide, only a small amount of BPA was formed, corresponding to about 2.5% of BPA glucuronide.

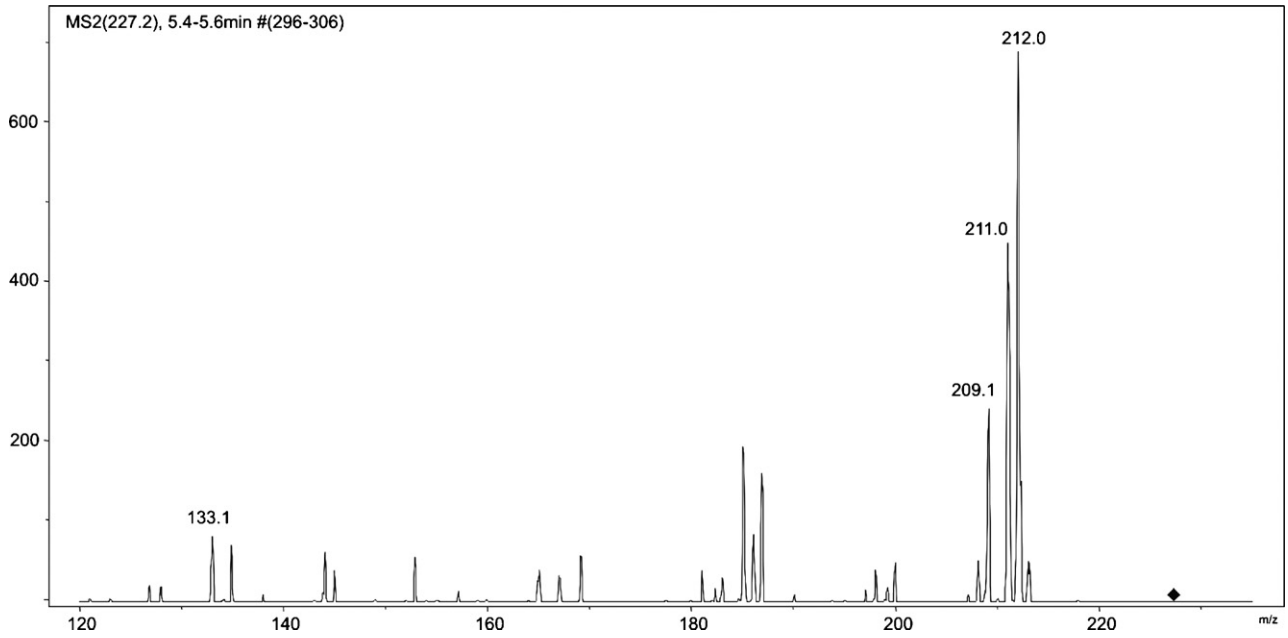

Fig. 3. Verification of the identity of BPA. MS/MS spectrum of the BPA peak (retention time 5.5 min) obtained after injecting an aliquot of a purified urine sample into HPLC system 2. The urine sample was subjected to enzymatic cleavage in the enzyme column followed by clean-up by immunoaffinity chromatography.

3.2.4. Quantitative determination of BPA

HPLC system 1 was calibrated by injecting seven standard solutions in the concentration range from 0.5 to 100 ng BPA/ml in mobile phase. A linear relationship was obtained between BPA concentration and peak area over the whole concentration range. The correlation coefficient was 0.9998 ($n = 7$). The detection limit (LOD, signal-to-noise ratio = 3) was found to be 1.0 ng BPA/ml.

The analytical performance of the whole method was assessed by applying the standard addition method. Since the purity of BPA glucuronide was not exactly known, only BPA was used for spiking the urine samples. Table 2 summarises the analytical data obtained by analysing one unspiked and three spiked urine samples. 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, calculated based on the LOD determined from the BPA standard calibration curve taking into account the enrichment factor by sample clean-up and the recovery assessed by the standard addition method.

3.2.5. BPA levels in urine samples

Since in a recently published paper BPA has been reported to migrate from artificial dialysers the analytical method developed in the present study was applied to investigate if there is any difference in the urinary BPA levels from healthy adults and dialysis patients. In contrast to urine samples from healthy adults which were collected in glass beakers, urine samples from dialysis patients were obtained and stored in plastic beakers. Those plastic beakers had to be tested with regard to BPA leakage in order to exclude systematic errors in the BPA concentrations obtained. Three urine samples with pre-determined free BPA concentrations were stored in plastic beakers at −20 °C for 10 days, the maximum time period from urine collection to sample analysis in the present study. After 10 days of storage the urine samples were found to contain the same BPA levels as determined immediately after sample collection, indicating that leakage of BPA from the urine collecting beakers did not occur.

Table 2
Analytical recovery of BPA in human urine

Spiking value (ng/ml)	Measured value (ng/ml)	Recovery (%)
0	0.63	
0.95	1.40	81.5
1.89	2.07	76.1
2.84	2.72	73.7
3.79	3.62	79.0

Table 3 summarises the data obtained by analysing urine samples from 10 dialysis patients and 12 healthy adults, e.g. concentrations of free and total BPA as well as the concentrations adjusted to creatinine. Fig. 4 shows the chromatograms of a purified urine sample from a healthy adult (sample 2, Table 3), (A) without and (B) including enzymatic cleavage of the BPA conjugates. In urine samples from healthy adults the medians of free and total BPA levels were 0.3 ng/ml (0.4 μg/g creatinine) and 1.1 ng/ml (1.0 μg/g creatinine), respectively, being in good agreement with previously published data. In a study focusing on the determination of urinary BPA levels in 394 adults of the USA, Calafat et al. reported the median of total BPA to be 1.28 ng/ml or 1.32 μg/g creatinine [20]. By determining BPA levels in 30 healthy Koreans, Kim et al. obtained free BPA levels in the range from 0.068 to 2.36 ng/ml and total BPA levels from 0.85 to 9.83 ng/ml [25]. Ye et al. determined the BPA concentrations in 30 human urine samples collected between 2000 and 2004 [21]. The concentrations of free BPA

Table 3
BPA levels in urine samples from dialysis patients and healthy adults

Sample	Total BPA (ng/ml)	Free BPA (ng/ml)	Creatinine (mg/ml)	Total BPA (μg/g creatinine)	Free BPA (μg/g creatinine)
Dialysis patients					
1	1.2	0.3	0.5	2.4	0.6
2	1.2	0.2	0.8	1.4	0.3
3	2.5	0.7	0.5	4.7	1.4
4	0.5	0.2	0.3	1.6	0.6
5	0.4	0.2	0.1	3.0	1.8
6	0.6	0.2	0.4	1.6	0.7
7	2.6	1.2	0.4	6.2	3.0
8	1.8	<LOD	0.5	3.3	<LOD
9	1.6	0.2	0.2	8.9	1.4
10	0.5	0.4	0.5	1.2	0.8
Healthy adults					
1	0.6	0.2	2.0	0.3	0.1
2	2.5	0.2	0.7	3.7	0.3
3	1.1	<LOD	2.4	0.5	<LOD
4	0.9	0.6	0.6	1.5	1.0
5	3.7	0.3	0.7	5.4	0.4
6	1.7	<LOD	4.0	0.4	<LOD
7	4.9	0.3	3.0	1.6	0.1
8	1.1	0.3	0.8	1.4	0.4
9	0.7	0.4	0.9	0.8	0.5
10	5.6	0.8	1.4	4.1	0.6
11	0.8	0.3	0.8	1.0	0.4
12	0.2	<LOD	1.0	0.2	<LOD

LOD = 0.2 ng/ml.

were below the LOD (0.3 ng/ml), the median of total BPA was 2.12 ng/ml.

In urine samples from dialysis patients the median of free and total BPA levels were 0.2 ng/ml (0.8 μg/g creatinine) and 1.2 ng/ml (2.7 μg/g creatinine), respectively, indicating that – without adjusting to creatinine – the medians of free and total BPA concentrations are very similar to the results obtained for healthy adults. Since the dialysis treatment significantly lowers urine creatinine concentrations, in dialysis patients the ratio of the concentrations of BPA and creatinine is higher than in healthy adults.

3.2.6. Column to column reproducibility

The experiments described above were carried out with enzyme and immunoaffinity columns packed with different batches of protein doped sol–gel glasses and the performance of the new columns evaluated by determining their recovery using standard solutions. The recoveries obtained with columns from different production batches did not differ significantly.

3.2.7. Stability of sol–gel columns

After having used a sol–gel enzyme column for cleavage of BPA conjugates in 25 urine samples, the cleavage efficiency of

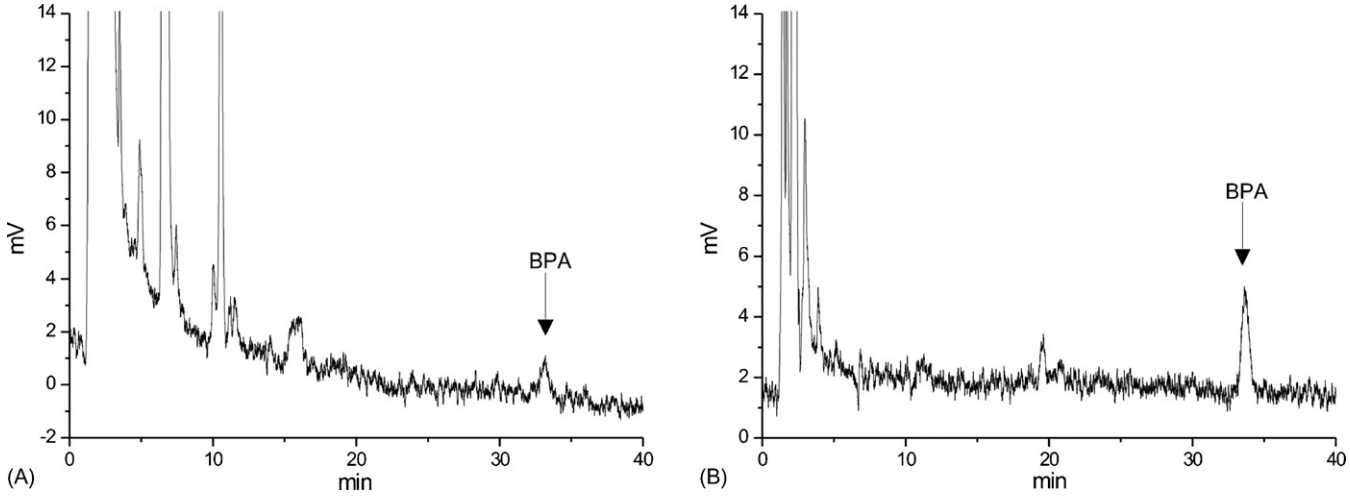

Fig. 4. Selectivity of the sample clean-up procedure. HPLC chromatograms of a purified urine sample from a healthy adult (sample 2, Table 3); (A) without enzymatic cleavage, concentration factor 10; (B) after enzymatic cleavage, concentration factor 2.

the column was checked by enzymatically hydrolysing one and the same sample by two methods, in the enzyme column and by adding glucuronidase and arylsulfatase to the sample solution. Cleavage in the enzyme column yielded the same BPA level as incubating the sample solution with the enzymes overnight, indicating that repeated use of the column did not decrease its cleavage efficiency.

After having purified a certain number of urine samples the recovery of BPA from the immunoaffinity column was determined by the use of a BPA standard solution. After the application of 13 urine samples the recovery was 100% and after 18 urine samples the recovery was decreased to only 76%.

4. Conclusion

By selecting the appropriate operating conditions, it was possible to make use of the different potentials of sol –gel generated enzyme and antibody columns in an on-line combination designed for the sample clean-up of urine samples. Combining a sol–gel column containing two enzymes, glucuronidase and arylsulfatase, and a column containing anti-BPA antibodies allowed a fast and highly selective sample pre-treatment procedure for the determination of BPA in urine samples.

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