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Application of liquid chromatography–mass spectrometry to the quantification of bisphenol A in human semen

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Abstract

The potential risks to human health and reproduction from the xenoestrogen bisphenol A (BPA) have not been well established. This is due in part to the absence of accurate analytical methods to quantify BPA in biological samples. In this study we establish an accurate, sensitive and selective analytical method for the quantification of BPA in human semen. To quantify BPA we compared the techniques of liquid chromatography–mass spectrometry (LC–MS) and enzyme-linked immunosorbent assay (ELISA). In addition we have taken steps to eliminate BPA contamination during sample extraction and preparation. Results show that the ELISA method gives an over-estimate of BPA concentration, which may be due, at least in part, to non-specific interactions with the BPA-antibodies. LC–MS gave much more accurate results and proved to be more sensitive with a detection limit of 0.5 ng ml^{-1} compared to 2.0 ng ml^{-1} by ELISA.

Keywords: Bisphenol A

1. Introduction

The endocrine system is a complex network that involves many tissues including the brain, pituitary gland, adrenal gland and reproductive system, all releasing hormones into the blood. Endocrine disrupting chemicals consist of synthetic and naturally occurring chemicals that affect the balance of hormonal functions in humans and animals. Some pesticides and industrial chemicals can affect animal physiology by mimicking the effects of endogenous hormones [1,2]. Several of these chemicals have been shown to have estrogenic activity both in vitro and in vivo [3–5]. One potential 'xenoestrogen' is bisphenol A (BPA), a chemical widely used in the manufacture of chemical products such as epoxy resins and polycarbonate [6,7]. Recent data have shown that BPA can bind to the oestrogen receptor inducing estrogen-dependent gene expression in various cell types, including those derived from breast cancer tissue [8–10]. In addition, studies in vivo have examined the potential health effects of BPA on reproduction and generation [11–13]. Thus, the potential effect of BPA on human health gives cause

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for concern and must be examined. There are, however, few studies in this area, although we have previously reported the presence of low levels of BPA in blood taken from healthy humans [14]. As the endocrine system is closely involved in the reproductive process we have examined levels of BPA in human semen, as it is a likely site at which xenoestrogens might exert their effect.

The aim of this study was to develop an accurate, selective and sensitive analytical method for the quantification of BPA in human biological samples. We compared two methods. Firstly, we examined an enzyme-linked immunosorbent assay (ELISA) offering a quick, inexpensive and simple protocol [15]. Second, we tested the liquid chromatography–mass spectrometry with electrospray ionisation (LC–MS) technique using a deuterium-labeled surrogate standard. In addition we examined the processes of sample collection and preparation.

2. Experimental

2.1. Reagents

Bisphenol A standard was purchased from Kanto Chemical Industries (Tokyo, Japan). BPA-d_{16} surrogate standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade acetonitrile for mobile phase was purchased from Wako Pure Chemical Industries (Osaka, Japan) as were all pesticide-grade solvents used in sample preparation. Purified water was obtained using a Milli-Q gradient A10 Elix with EDS polisher system (Millipore, Bedford, MA, USA). The EDS polisher is a new filter purchased from Millipore, Japan, that we estimated would give non-contamination of BPA in pure water [16]. Therefore, distilled water purified by this system is useful for analysis of trace levels of BPA in a variety of samples.

2.2. Semen samples

Semen samples were obtained from 41 healthy volunteers aged 18 to 38 (25.0±5.5). All samples were stored at −80 °C.

2.3. BPA enzyme-linked immunosorbent assay

A competitive BPA enzyme-linked immunosorbent assay (ELISA) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and used according to the manufacturer's instructions. Briefly, the sample was pre-conditioned with 10% methanol and mixed with the enzyme-labeled BPA solution and 100 μl transferred to separate wells of a 96-well plate coated with anti-BPA antibodies. The plate was then incubated at room temperature for 60 min. Un-bound antibodies were removed by washing three times with the wash-buffer. Then 100 μl per well of luminescent solution was added and the plate incubated in the dark for 30 min, after which the reaction was inhibited by the addition of stop solution. The absorbance was then measured at 450 nm using a Bio-Rad 550 Micro-Plate Reader.

2.4. Liquid chromatography–mass spectrometry sample preparation

A reported method for the determination of BPA in human serum was modified for extraction of trace levels of BPA from human semen [14]. Samples were extracted using solid-phase extraction (SPE) cartridges (Shodex SPEC EDS-1 from Shoko, Japan). In order to prevent the analytes taking their ionic forms the samples were acidified to pH 3.0 with 1.0 *M* HCl. Before extraction the SPE columns were pre-conditioned with 15 ml of methanol followed by the addition of 3.0 ml of water adjusted to pH 3.0 with HCl. From each semen sample 1.0 ml was acidified and added to 2.0 ml of water and BPA-d_{16}. The samples were passed through the SPE cartridges which were then washed with 3.0 ml of 10% methanol in water. Then 3.0 ml of methanol introduced at low flow-rate was used to elute the retained compounds. The solution was dried under a stream of nitrogen at 40 °C prior to re-suspension in 500 μl of methanol. Samples were then subjected to LC–MS as described below.

2.5. Liquid chromatography–mass spectrometry measurements

LC–MS was performed using a Senshu Pak PEGASIL ODS (3 μm, 2×150 mm) reversed-phase

column (Senshu Scientific, Tokyo, Japan) in an Agilent 1100 MSD-SL system linked to an electrospray (ES) ionisation interface (Agilent Technologies, Palo Alto, USA).

The injection volume was 5 μl and the column oven was maintained at 40 °C for LC. This separation was carried out using a mobile phase from 0.01% acetic acid in water (Mobile phase A) and pure acetonitrile (Mobile phase B). The gradient mode was as follows; 0–12 min at 40% Mobile phase B, then 12–14 min linearly increasing from 40 to 100% Mobile phase B, which was held. The flow-rate was 0.2 ml min^{-1}. The working conditions for electrospray ionisation MS were as follows: the drying nitrogen gas temperature was set at 350 °C and the gas introduced into the capillary region at a flow-rate of 12 l min^{-1}; the capillary was held at a potential of 3500 V relative to the counter electrode for the negative-ion mode. The fragmentor voltage was 140 V during the chromatographic run. When working in the selected ion monitoring (SIM) mode we assigned the *m/z* 227 and 241 ions as the $[M\text{-}H]^-$ of BPA and the $[M\text{-}D]^-$ of BPA-d_{16}, respectively. Here, BPA-d_{16} (MW 244) was transformed to BPA-d_{15} (MW 243) in water. For this reason, *m/z* 241 was assigned to the $[M(BPA\text{-}d_{15})\text{-}D]^-$ in negative-ion detection.

Standard solutions of BPA were prepared in methanol and added to a fixed concentration of BPA-d_{16}; this resulted in a calibration curve covering the concentration range 2.25–450 pg. Quantitative analysis was performed using selected ion monitoring in order to maximize sensitivity. BPA concentrations were calculated relative to BPA-d_{16} standards that were added to the samples prior to extraction, giving a final extract concentration of 100 ng ml^{-1}. Eight-point calibrations were performed daily for all analytes with internal standards.

3. Results and discussion

3.1. Detection of bisphenol A by enzyme-linked immunosorbent assay

We examined the ELISA detection of known quantities of BPA spiked into pure Milli-Q water. Results were found to be linear over the range 0–130 ng ml^{-1} with correlation coefficients (*r*) better than 0.999 (Fig. 1). We also found that the IC_{50} was 29.0 ng ml^{-1} and that the limit of quantitation (LOQ) was 2.0 ng ml^{-1} (IC_{10}).

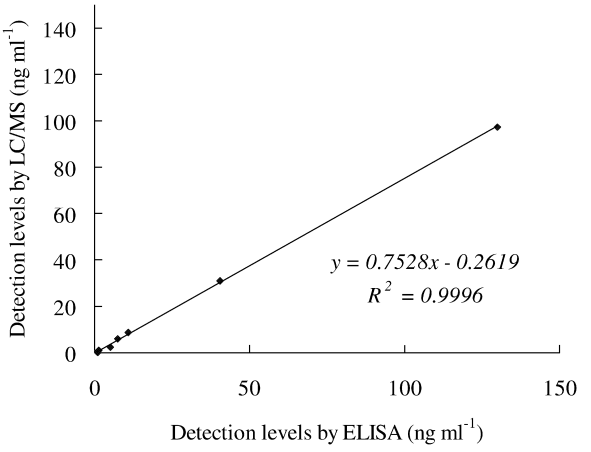

Fig. 1. Comparison between ELISA and LC–MS for determination of BPA in water.

3.2. Liquid chromatography–mass spectrometry validation and calibration

In the mass spectral analysis using ES-MS with flow through injection analysis of BPA standard solutions (10 μg ml^{-1}), the *m/z* 227 ion which was assigned as the $[M\text{-}H]^-$ ion was observed as the main peak. The most important parameters affecting LC–MS for determination of compounds are the fragmentor voltage and the mobile phase effect. In order to establish the optimum fragmentor voltage for the detection of BPA, the *m/z* 227 signals for BPA were investigated versus fragmentor voltage. The mobile phase effect was described previously [17]. The main *m/z* signals showed a maximum in 0.01% acetic acid at 140 V for BPA.

The method detection limit (DL) was calculated as 3× standard deviation of the analyte concentration determined in the filed blank. The calculated DL was 0.1 ng ml^{-1} for LC–MS detection levels. In addition, the LOQ was 0.5 ng ml^{-1} in semen samples according to signal response/background-noise-level=10. LC–MS with SIM calibration standard was used to investigate the linearity of the method.

Peak area ratios with respect to internal standard were plotted. The response was found to be linear in the validated range with correlation coefficients (r) better than 0.999. The method yields a highly precise determination of standards and might be applied to the detection of trace amounts of BPA in semen.

3.3. Recovery from solid-phase extraction and liquid chromatography–mass spectrometry

The absolute recovery from the method is shown in Table 1. Average recovery of BPD-d_{16} was 71.2%. In addition, an average recovery of 100.5% (RSD=4.7%) was found for 1.0 ng ml^{-1} of BPA. Accuracy of the method was determined as recovery relative to the internal standard.

3.4. Accuracy of bisphenol A quantification

Analysis of trace levels of BPA in biological samples is complicated by contamination, particularly by leaching from plastics. Thus, care must be taken to control for this during experiments and where possible the source of contamination must be eliminated. For example, for the collection of human semen a plastic cup is used that may contaminate the sample. We investigated this potential source of contamination using a protocol previously reported for the determination of additives in plastic [18]. Briefly, BPA in the plastic cup was extracted by soaking overnight at 40 °C with a solution of cyclohexane and 2-propanol (1:1). The solution was dried, re-dissolved in methanol and then analyzed by LC–MS. In this case BPA was not detected (<4.0 ng g^{-1}) so the sampling cup was not a source of contamination in these experiments. We have previously reported on BPA contamination of the Milli-Q water and the SPE columns [14,16].

Table 1
Recovery test of BPA is spiked human semen

BPA calculated relative to I.S. Recovery (%)	BPA-d_{16} Recovery (%)
100.0	71.9
107.5	73.2
108.0	72.1
97.1	71.3
98.3	69.4
92.3	72.2
Average 100.5 (RSD: 4.7%)	Average 71.2

Spike amount: BPA 1.0 ng ml^{-1} and BPA-d_{16} 50 ng ml^{-1} in semen.

3.5. Measurement of bisphenol A in human semen

We examined 41 semen samples for the presence of BPA using ELISA and LC–MS. Using ELISA, results from the semen samples ranged from no detection to 12.0 ng ml^{-1}, with the average being 5.1 ng ml^{-1}. Measurements made by LC–MS failed to detect BPA in any samples (i.e. <0.5 ng ml^{-1}). However, trace levels of BPA under LOQ were detected (Fig. 2).

These data suggest that the LC–MS method can detect the absence of or very low level of exposure to free BPA in human semen. In addition, we have demonstrated that the ELISA method may give erroneous values which may be due to non-specific binding to the antibody, leading to an over-estimation of BPA concentration.

4. Conclusions

The development of analytical techniques for the accurate quantification of BPA in humans is a vital step in understanding its effects on human health and reproduction. Here we demonstrate that LC–MS is the method of choice for the accurate analysis of BPA levels in human semen and that this may be extended to include other human biological samples. We also show that it is necessary for control measurements to be taken from sampling containers and water used in the sample preparation. Further studies will be conducted to examine BPA metabolism [19].

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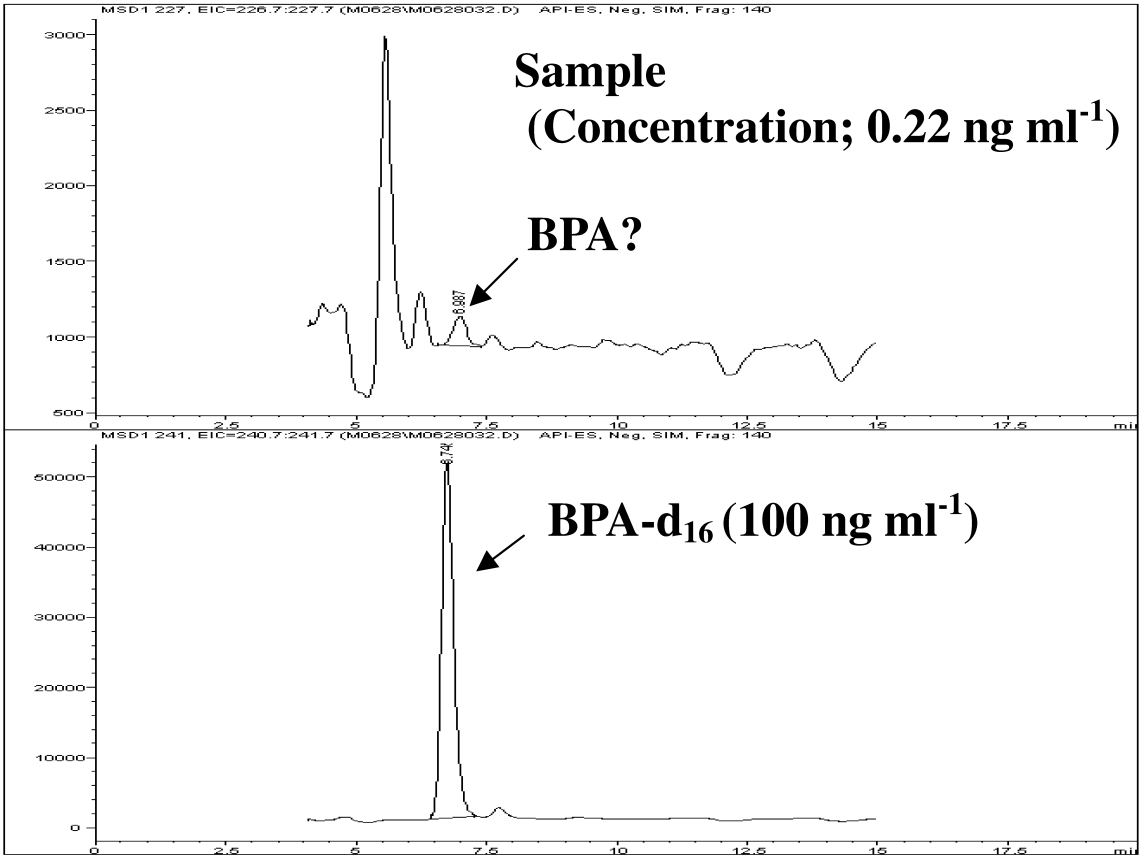

Fig. 2. LC–MS/SIM chromatograms of BPA and BPA-d_{16} in human semen.

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