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Bisphenol A-glucuronide measurement in urine samples

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ABSTRACT

Bisphenol A (BPA), is one of the most abundant endocrine disruptors that are present in our environment, and has been repeatedly detected in most human biological samples. As it has been suggested that part of the BPA measured in human samples is due to contamination during samples collection or laboratory measurements, we have developed a specific radioimmunoassay for the measurement of BPA-glucuronide (BPA-G), the main endogenous metabolite of BPA in urine. We used a polyclonal anti-BPA antibody which has a 95% cross reactivity with BPA-G, and insignificant cross reactivity with most analogous BPA phenolic structures. To eliminate unconjugated BPA from urine samples, an extraction step with dichloromethane was required. The method proved to be valid, precise and accurate in the range of 0.05 μg/L to 5 μg/L. With this method, we measured BPA-G in 163 urine samples from a hospital population. We detected BPA-G in all samples, with mean values of 4.64 μg/L. In conclusion, the present radioimmunoassay is a useful tool for the screening of BPA exposure in human populations encompassing the problem of eventual contamination from laboratory manipulation.

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1. Introduction

Bisphenol A (BPA) is one of the most abundant endocrine disruptors present in our environment [1]. The ingestion of food is considered as the main route of BPA exposure in humans, which was estimated by the Scientific Committee of the European Commission to be in the range of 0.4 to 1.6 μg/kg BW/day [2]. However, other sources of contamination also of concern include indoor air, dust, dental sealant, thermal printing paper, and implanted medical devices [3–6]. As a result of the widespread use of this product, human exposure to BPA is continuous. It is therefore conceivable that BPA is detected in more than 90% of urine samples from different human reference populations and is present in most biological samples [1,7]. Recently, several studies have reported a significant association between increasing urinary concentration of BPA and higher incidence of several chronic diseases, such as diabetes, cardiovascular diseases, liver enzyme abnormalities, obesity, and polycystic ovary syndrome [8–13]. Pharmacokinetic studies have documented that BPA is rapidly metabolized in BPA monoglucuronide whose metabolic clearance rate is 6 h [14,15]. Because of the fast urinary excretion of BPA glucuronide, unconjugated BPA concentrations are expected to be relatively low. In contrast, most studies reported that the serum concentration of unconjugated BPA ranged from 0.2 to 20 μg/L [16–19]. It has therefore been hypothesized that such levels may not reflect only exposure to BPA, but also contamination from the consumables used during sample draw [20] and laboratory analyses [21], and/or from the deconjugation of BPA metabolites during storage [22]. Indeed, during the developpement of our plasma BPA assay, we have observed that some medical device can be a source of BPA contamination. Most of the methods detecting the total urinary BPA (unconjugated and conjugated forms of BPA) after enzymatic hydrolysis, require laborious extraction, clean-up and/or derivatization steps that provide numerous potential sources of contamination. To overcome the dilemma of BPA contamination during sample handling, we developed an assay for the direct measurement of the conjugated BPA glucuronide (BPA-G), without hydrolysis. Because of its liver production, BPA-G represents the BPA actually ingested by the subject. This assay uses the anti-BPA antibody and the tracer previously prepared for plasma measurement [19], and requires only the elimination of unconjugated BPA by one solvent extraction step. It is a specific,

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reproducible and very sensitive method, with few sample workup, allowing the evaluation of human exposure to BPA in a large numbers of samples.

2. Materials and methods

2.1. Materials

The polyclonal anti-BPA antibody was obtained by E. Mappus and C.Y. Cuilleron (INSERM U863, Lyon), by immunization with BPA carboxymethylether coupled to BSA. BPA was labeled with ^{125}I by the chloramine T method, and purified by HPLC on C18 column [19].

Bisphenol A β-D-glucuronide was purchased from Santa Cruz Biotechnology, Inc, and BPA from Aldrich. [^{125}I] NaI, volumic activity 3.7 GBq/mL was obtained from PerkinElmer. Ethyl acetate was of quality Pestipur and all other chemicals were of analytical grade.

2.2. RIA procedure

The measurement of BPA-G concentrations was performed by applying on diluted urine samples the assay previously developed for plasma [19]. In brief, BPA-G standard was dissolved in water to produce a stock solution of 1 μg/L. For each assay, the solution was diluted to provide standards over a range of 0.16 to 5 μg/L. BPA-G was measured after elimination of free BPA as follow: standards (500 μL) or urinary samples (100 μL urine and 400 μL water) were pipetted into conical tubes. After addition of 500 μL dichloromethane, agitation on a vortex, and centrifugation, 100 μL of the upper phase was transferred in duplicate into polystyrene tubes for the RIA. Then, 100 μL of ^{125}I–BPA tracer (~20,000 cpm) and 100 μL of antibody solution, diluted 5000-fold in assay buffer (PBS and gelatin 1 g per liter) were added. The final volume of 500 μL was completed for each sample with assay buffer. Samples were incubated for 2 h at room temperature. Then, 500 μL charcoal suspension (0.54 g Dextran T70 and 5.4 g charcoal Norit A per liter in assay buffer) at 4 °C were added, and after 10 min incubation at 4 °C and centrifugation for 10 min, the radioactivity of the supernatant, containing the BPA-G bound to antibody, was counted in a gamma-counter (Cobra, PerkinElmer).

Urinary unconjugated BPA concentrations were measured after an extraction step as previously performed for plasma [19]. A BPA solution was diluted in water to provide standards over a range of 0.08 to 5 μg/l. Then, 0.25 ml urine samples and standards were pipeted into glass tubes containing 0.75 ml of NH4OH 5% solution. After the addition of 2.5 ml ethyl acetate (quality Pestipur) and agitation on a Vortex, 2 ml of upper organic phase containing unconjugated BPA was transferred into a glass tube and evapored to dryness under a nitrogen stream. The dry residue was dissolved in 0.5 ml of assay buffer. Then RIA was performed as the BPA-G RIA.

2.3. Subjects and sample collection

For an initial screening of BPA-G concentrations in urines, we measured BPA-G concentrations in 163 urine samples from subjects coming to our hospital for consultation. Spot urine samples were collected between 08.00 and 12.00 am, in containers that are controlled to not release BPA.

To study the 24 h BPA-G excretion profile, we collected fractionated urine samples (throughout the day) from 14 other subjects. During this collection, samples were stored at room temperature. At the end of the collections, urine samples were aliquotted and stored at −20 °C until analyses. Exact volume for each urine sample was carefully recovered to express BPA-G excreted as μg.

We measured conjugated and unconjugated BPA in urine samples from 24 subjects to estimate the correlation between BPA-G and total BPA. In addition to urine samples, we also collected plasma from 67 patients that were sent to our clinical analysis laboratory, at the same time, to explore the correlation between urinary BPA-G and plasma unconjugated BPA concentrations.

2.4. Analytical performance

The detection limit was defined as the concentration giving a displacement of 3 standard deviations (SD) from maximum binding counts (n=10). To establish inter assay precision, five replicated standard curves were constructed using 3 control urine samples containing 0.2, 0.9 and 1.7 μg/L of BPA-G. To ascertain the intra-assay precision, the control urines were also assayed in 6 replicates, in one single run.

Recovery tests were carried out by adding 0.31, 1.25 and 5.00 μg/L of BPA to human urine samples. The effectiveness of the unconjugated BPA elimination by the dichloromethane step was quantified by assaying urine samples loaded with increasing concentrations (0.15 to 10 μg/L) of unconjugated BPA.

Stability of BPA-G was assessed by assaying BPA-G after eight freezing/thawing cycles on two samples stored at −20 °C, and on 6 urine samples left at room temperature and assayed at t_0, t_1 (t_0+8 h) and t_2 (t_0+24 h).

As a comparison, concentrations of BPA-G by immunoassays on 32 urine samples were compared to measurements of total BPA by gas chromatography/mass spectrometry (GC/MS). Measurements of total BPA by GC/MS were performed at the laboratory of the Biomarkers Group, International Agency for Research on Cancer, Lyon, France. In short, d_{16}-BPA was added to 1 ml of urine sample that was hydrolyzed with arylsulfatase/ß-glucuronidase for 20 h at 37 °C. Hydrolyzed samples were extracted on SPE columns, and total BPA recovered with ethyl acetate. Samples were then dried, reconstituted with ethanol/water (1/1 v/v) and injected into a high performance liquid chromatography system for purification. The fraction containing BPA+d_{16}-BPA was then collected, and then dried under N_2 at 40 °C. Then, samples were derivatizated with BSTFA–TMCS, evaporated under N_2 at 40 °C, then reconstituted in hexane and injected in the GC/MS system (DSQ Gas Chromatograph Mass Spectrometer Thermo-Finnigan, USA) for detection. The detection limit of the method is 0.05 μg/L, intra- and inter-batch CVs were 4.5 and 10.8 respectively, for a concentration of 0.8 μg/L.

3. Results

3.1. Assay performance

Our polyclonal anti-BPA antibody has a 95% cross reactivity with BPA-G, and demonstrates negligible cross reactivity with most analogous BPA phenolic structure [19]. No cross reactivity could be detected with glucuronic acid or steroid-glucuronide (estrone-glucuronide).

The range of standard curve of the BPA-G RIA was from 0.16 to 5 μg/L, with an IC50 of 0.30 μg/L, and a detection limit of 0.05 μg/L (Fig. 1). Dilution tests, with urine samples had a linear profile (r=0.97).

The intra- and inter-assay coefficients of variation were 13% and 14% respectively at a concentration of 1 μg/L, and 6% at a concentration of 4 μg/L. The yields of recovery, measured using urine samples spiked with BPA-G at concentrations of 0.30, 1.25 and 5 μg/L, were 106 ± 7%, 116 ± 4% and 92 ± 6% respectively.

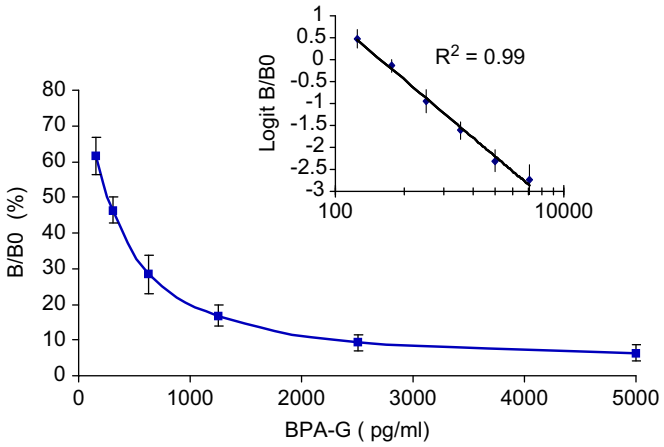

Fig. 1. Composite standard curve of BPA-G ($n=6$).

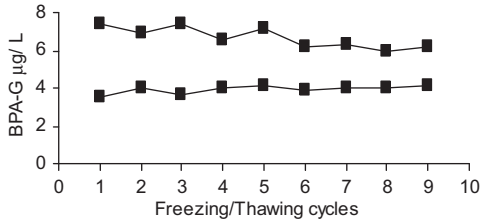

Fig. 2. BPA-G concentrations in 2 urine samples after 8 freezing/thawing cycles.

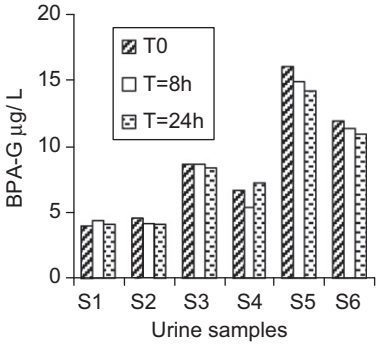

Fig. 3. BPA-G concentrations in 6 urine samples left at room temperature and assayed at t_0, t_1 (t_0+8 h) and t_2 (t_0+24 h).

The dichloromethane step eliminates 90% of unconjugated BPA. As shown in Figs. 2 and 3, BPA-G concentrations in urine samples were stable after several freezing/thawing cycles and after being at room temperature for 24 h.

A Pearson's correlation coefficient of $r=0.86$ was observed between BPA-G concentrations measured by RIA, and total BPA measured by GC/MS in 32 urine samples.

3.2. Human exposure to BPA-G

The geometric mean of BPA-G concentrations measured on morning spot urine samples from 163 subjects was 4.64 µg/L, corresponding to 2.62 µg/L BPA (Fig. 4). The 24-h excretion profile of BPA-G showed a great intra- and inter-person variability (Table 1). However, the BPA-G excretion during 24H00–8H00 correlated with total daily BPA-G excretion ($r=0.93$). There was a highly significant correlation ($r=0.97$) between the levels of total BPA (unconjugated BPA and BPA-G) and BPA-G, with BPA-G representing 79 ± 9% of urinary BPA (Fig. 5).

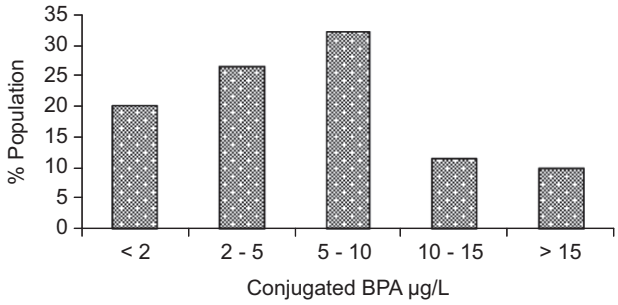

Fig. 4. Distribution of BPA-G concentrations in 163 urine samples.

Table 1
Urinary excretion of BPA-G over 24 h in 14 subjects.

BPA-G (µg)						
	8–12 h	12–16 h	16–20 h	20–24 h	24–8 h	Total 24 h
P1	1.06	1.49	1.61	1.58	1.96	7.70
P2	0.78	0.58	4.02	4.36	5.53	15.27
P3	2.63	1.47	0.46	1.93	3.77	10.26
P4	0.38	0.41	0.57	0.65	0.11	2.12
P5	0.71	1.72	1.64	0.95	1.07	6.09
P6	0.15	3.57	0.43	2.68	7.48	14.31
P7	1.09	1.34	0.79	1.56	5.72	10.50
P8	0.49	0.62	0.63	1.03	2.00	4.77
P9	3.58	2.78	1.72	2.06	4.77	14.91
P10	0.99	0.95	0.83	1.08	1.38	5.23
P11	0.46	0.47	0.68	0.52	0.76	2.89
P12	0.16	0.35	0.33	0.33	0.17	1.34
P13	0.59	0.70	0.63	1.51	1.02	4.45
P14	0.90	0.96	1.13	1.09	1.55	5.63

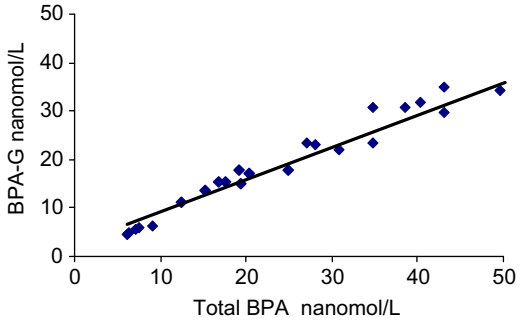

Fig. 5. Correlation between urinary total BPA and BPA-G concentrations.

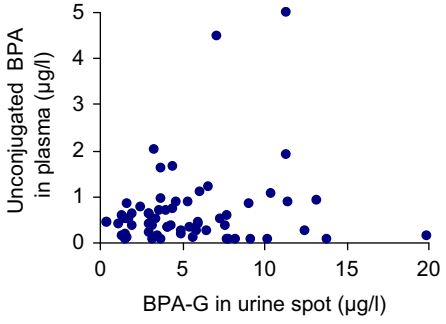

Fig. 6. Plasma BPA concentration versus urinary BPA-G concentration, for samples. collected at the same time ($n=67$).

No correlation was found between unconjugated BPA concentrations measured in plasma samples and BPA-G concentrations measured in urine spot samples collected from the same subjects ($n=67$) at the same time of the day (Fig. 6).

4. Discussion

An accurate exposure assessment of BPA in different populations is essential to explore the potential risk of BPA on human health. It is commonly agreed that the measurement of conjugated or total BPA concentrations in urine samples is representative of BPA environmental human exposure [21]. However, controversy over possible contamination of biological samples, during collection, or laboratory measurements persists. Therefore, we have adapted our radioimmunological assay, initially developed for measuring unconjugated BPA in plasma, to the measurement of BPA-G in urine. A step of solvent extraction was performed to remove free BPA from the samples. The recent availability of the monoglucuronide form of BPA as standard allowed us to test the cross reactivity of our polyclonal anti-BPA antibody to BPA-G. The antibody used was raised by immunization with BPA carboxymethylether coupled to BSA, and had a high specificity for the central part of the BPA molecule. As expected, our antibody had a 95% cross reactivity with BPA-G, but in contrast, showed negligible cross reactivity with most analogous BPA phenolic structures.

The high sensitivity of our assay allowed accurate BPA-G measurement for concentrations ranging from 0.16 to 5.0 μg/L. Because of the relatively high concentrations of BPA-G in some urine samples, a dilution step may be needed to correctly measure BPA-G within the range of the standard curve of our immunoassay. The limit of detection of our assay is comparable to that obtained by using GC–MS or LC–MS/MS for total BPA [23]. BPA-G concentrations measured by RIA and total BPA concentrations measured by GC/MS on 32 urine samples were comparable ($r=0.86$). With our RIA, good correlation was observed between total BPA and BPA-G levels in urine samples ($r=0.97$), which strongly suggests that there was no external contamination of the urinary samples during collection and assay. As reported in the literature, the majority of ingested BPA is excreted in urine as glucuronide conjugates while only a minor amount is excreted in the unconjugated form. However, other sources of contamination that can occur through non-oral pathways could explain the presence of unconjugated BPA in urine [21].

We have shown the stability of BPA-G in urine samples at room temperature, and after several freezing/thawing cycles. The results support that urine samples collected for analysis could be kept at room temperature for 24 h before being aliquotted and stored at −20 °C, and even after several freezing/thawing cycles.

We have performed a preliminary screening of urinary BPA by measuring BPA-G in urine samples from unselected patients referred to our outdoor clinics. We found urinary BPA-G within the range of concentrations previously reported by others studies [23]. In addition to the large between-subject variability, we also observed large within-subject variability as previously reported [24]. The overall temporal variability of BPA is probably due to the pharmacokinetic properties of BPA, as well as to changes in exposure driven by lifestyle factors and/or diet through the day and across days. However, the results of our study show that BPA-G excretion, measured in urine samples collected between 12 pm and 8 am seems to be representative of the total daily excretion. Therefore, despite a large within subject variation, a single urinary spot could be moderately representative of exposure overtime, and could be used for assessing BPA exposure in large-scale epidemiological studies [24].

5. Conclusion

We have developed an immunoassay that measures only the conjugated form of BPA, in order to limit the risk of misinterpretation of human BPA exposure because of the potential BPA contamination during sampling collection or laboratory analyses. This assay is easy, specific, relatively fast, and requires a minimal volume of urine. It can therefore be used for the evaluation of human exposure to BPA in a large numbers of samples for epidemiological studies.

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