Journal of Exposure Analysis and Environmental Epidemiology (2001) 11, 323–328


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Measurement of bisphenol A levels in human urine

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We report a new approach for assessing human exposure to bisphenol A (BPA) by measuring BPA in urine after enzymatic deglucuronidation. This method involves addition of $^{13}C_{12}$-labeled BPA, enzymatic deconjugation, solid-phase extraction, and derivatization with pentafluorobenzyl bromide. The product of the derivatization is separated by gas chromatography followed by mass spectrometric detection using negative chemical ionization and selected ion monitoring. Using this analysis method, urine samples fortified with both a constant level of labeled BPA and a range of unlabeled BPA levels (0.27–10.6 ng/ml) demonstrated constant percentage recovery. In addition, a range of urine sample volumes (0.25–10.0 ml) with constant amounts of added internal standard produced a linear response (r^2=0.99). The method limit of detection was 0.12 ng/ml. This method was validated by duplicate analyses using gas chromatography coupled to a high-resolution mass spectrometer. *Journal of Exposure Analysis and Environmental Epidemiology* (2001) **11,** 323–328.

Keywords: *biomonitoring, bisphenol A, GC/MS, human, pentafluorobenzyl bromide, solid-phase extraction, urine.*

Introduction

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is an industrial chemical used in the production of polycarbonate plastics, epoxy resins, and other products. Products potentially containing BPA include dental sealants and fillings, polycarbonate bottles, powder paints, food and drink containers, as well as beverage and food can linings. Prior to 1994, BPA could also be used as an "inert" ingredient in pesticide formulations. BPA has also been shown to be present in the natural environment. In natural water systems, BPA levels vary from <0.01 to 1.9 μg/l and have a half-life of 3–5 days (Matsumoto et al., 1977; Staples et al., 1998).

The relative importance of various sources of human exposure is poorly understood and vigorously debated. BPA appears to leach from lined cans and polycarbonate bottles. The rate of this leaching is under debate (Brotons et al., 1995; Hoyle and Budway, 1997). In addition, the leaching of BPA from dental sealants and fillings has been reported, but these results are also the subject of debate (Olea et al., 1996; Ashby, 1997; Imai, 1999).

When information is lacking about sources of exposure, internal dose measurements (such as monitoring markers in a biological tissue such as serum, urine or feces) must form the basis of human exposure assessment (Pirkle et al., 1995). BPA exposure in humans has not been studied except for one report of serum BPA levels in people. In this study, measurable levels of BPA ranging from <0.1 to 1.6 ng/ml were found in human serum samples (Sajika et al., 1999).

The usefulness of internal dose data can be limited by a lack of information on compartmentation, metabolism, and excretion. The compartmentation, metabolism, and excretion of BPA in humans have not been studied. In rodents, BPA is predominately excreted unmetabolized in feces with a serum half-life of less than 1 h (Pottenger et al., 2000). The other major metabolite excreted, glucuronidated BPA in urine, represents 15–34% of the original dose. BPA is also excreted as the urinary sulfate but at very low levels (<3%). Total urinary excretion may require as much as 2 days (Knaak and Sullivan, 1966; Pottenger et al., 2000). Differing excretion rates by gender and by route of administration were also found in these studies.

In preparation for a population-based human exposure study, we reviewed the literature for current methods for analyzing BPA. We found the methods possessed limitations or were not applicable to human biological samples (Gandara et al., 1993; Del Olmo et al., 1999) except for two techniques described in the previously mentioned study

1. Abbreviations: BPA, bisphenol A; EI+, positive electron ionization; GC/HRMS, gas chromatography coupled to high-resolution mass spectrometric detection; GC/MS, gas chromatography coupled to mass spectrometric detection; Hg, mercury; m/z, mass to charge ratio; NCI, negative chemical ionization; SPE, solid-phase extraction; TMS, trimethylsilyl

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Received 28 November 2000; accepted 25 April 2001.

(Sajika et al., 1999). These techniques were analyses of BPA in serum. Assuming the rodent findings apply to humans, measurement of BPA in serum provides information only about very recent exposure (1–3 h), whereas urinary BPA glucuronide levels are a longer lived biomarker (12–48 h).

Due to the difficulty of obtaining and extracting human fecal samples, the ease of urine collection, and the short half-life of BPA in serum, we developed a method to quantify BPA metabolites in human urine. This method extends a similar method to analyze BPA in serum (Yoshimura et al., 2000, unpublished). This new method involves enzymatic deconjugation, solid-phase extraction (SPE), derivatization with pentafluorobenzyl bromide and gas chromatography with mass spectrometric detection (GC/MS). This method is based on previous methods involving pentafluorobenzyl bromide derivatization (Daneshvar and Brooks, 1988; Jacobsson et al., 1988; Hofmann et al., 1990). Confirmation of the GC/MS method and identification of the final derivatized product were performed using gas chromatography coupled to a high-resolution mass spectrometer (GC/HRMS). Preliminary data suggest that the sensitivity of this method will allow measurement of background levels in urine from non-occupationally exposed humans.

Methods

All reagents and solvents were analytical grade unless specified otherwise (Burdick and Jackson, Muskegon, MI). All glassware was rinsed with hexane followed by acetone.

An aliquot (usually 1 ml) of urine was spiked with 20 μl of $^{13}C_{12}$-ring-labeled BPA (75.7 ng/ml, Cambridge Isotope Laboratories, Andover, MA). To samples requiring deglucuronidation, β-glucuronidase (*E. coli*, 5 μl, 200 U/ml, Roche Biochemical, Mannheim, Germany) was added followed by incubation at 37°C for 90 min. This treatment is sufficient to deconjugate the glucuronides of both 5-methylumbelliferone and phthalate monoesters (Blount et al., 2000). After enzymatic treatment, 1 ml of formic acid (32%; EM Industries, Gibbsville, NJ) was added to the

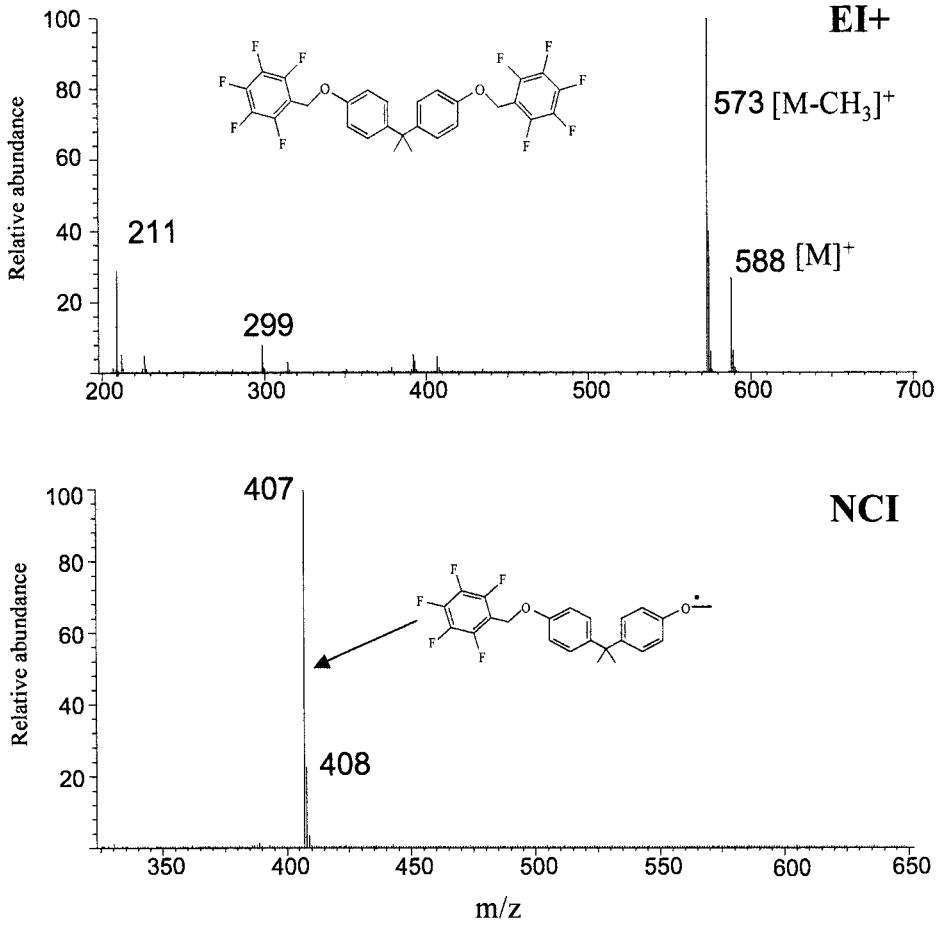

Figure 1. Positive electron ionization (EI+) and negative chemical ionization (NCI) mass spectra of GC peak eluting at 26.4 min.

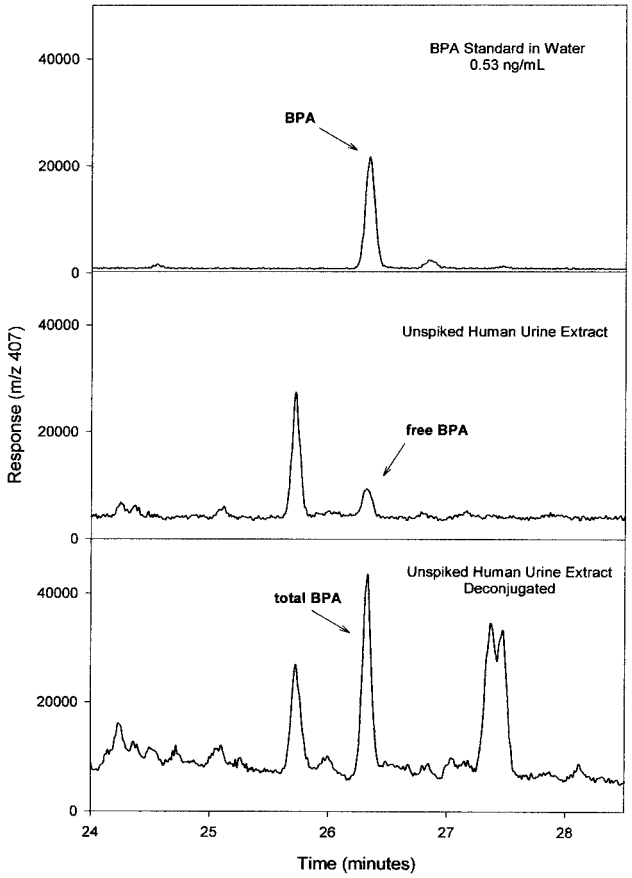

Figure 2. GC chromatograms. The human urine extracts are identical except for undergoing or omitting enzyme treatment. (a) BPA standard in water (0.53 ng/ml); (b) unspiked human urine extract; (c) unspiked human urine extract — deconjugated.

sample and then buffered with ammonium acetate (250 μl, 1 M, pH 6.5, Sigma, Milwaukee, WI).

An octyldecyl SPE column (500 mg, J.T. Baker, Phillipsburg, NJ) was prepared by washing under partial vacuum (5 mm Hg) with 15 ml of methanol followed by 5 ml of water. Water was left just above the head of the column. The spiked sample was added and pulled with partial vacuum (5 mm Hg) onto the column until it was even with the top of column. The column and sample stood for 10 min without vacuum. Then 5 ml of water/methanol (90:10 v/v) was added to the column and pulled through with partial vacuum (5 mm Hg). The column was allowed to go dry with partial vacuum (15 mm Hg). Clean tubes were placed under the manifold and 8 ml of methanol was added to the column and pulled through with partial vacuum (5 mm Hg). Samples were evaporated just to dryness under a stream of nitrogen using a Turbovap (55°C, 5 psi, 20 min; Zymark, Framingham, MA).

Methylene chloride (1 ml; Calendon Laboratories, Georgetown, Ontario, Canada) was added to the sample tubes and mixed. To the sample tubes, we added 0.5 ml of 0.1 M tetrabutylammonium hydrogen sulfate (Eastman Kodak, Rochester, NY), 0.5 ml of 0.2 M sodium hydroxide (Mallinkrodt Baker, Paris, KY), and 20 μl of α-bromo-2,3,4,5,6-pentafluorotoluene commonly called pentafluorobenzyl bromide (Supelco, Bellefonte, PA). Tubes were mixed for 25 min at room temperature using a mechanical mixer inverting the tubes about twice per minute.

After derivatization, the tubes were centrifuged for 1 min at 1600 rpm. The methylene chloride layer was removed by pipet and placed in a clean tube and concentrated just to dryness under a steam of nitrogen (20 min, 55°C; Turbovap; Zymark). To the tube, 2,2,4-trimethylpentane (0.5 ml, Mallinkrodt Baker) was added and mixed. The 2,2,4-trimethylpentane solution was transferred into clean GC vials.

The derivatized samples were analyzed using a single quadrupole GC/MS (HP 6890 and 5973, Agilent, Wilmington, DE). The injection volume was 2 μl in the splitless mode with the injector temperature of 245°C. The injector purge time was 1 min. The column was a DB-5, 30 m in length with a film thickness of 0.25 μm and internal diameter of 0.25 mm (J&W Scientific, Folsom, CA). The carrier gas was helium with a constant flow of 1 ml/min. The oven temperature program was 60°C initially and a single step to 270°C at 15°C/min. The total run time was 31 min.

The MS used negative chemical ionization (NCI) with methane as the reagent gas with a pressure of 1.8×10^{-4} Torr. The source temperature was 260°C. Later experiments suggest that 270°C improves the ion abundance. The transfer line was heated to 280°C. The solvent delay was 5.6 min. The native BPA signal was monitored in selected ion mode at mass to charge ratio (m/z) 407 and the $^{13}C_{12}$-labeled BPA signal was monitored at m/z 419. These

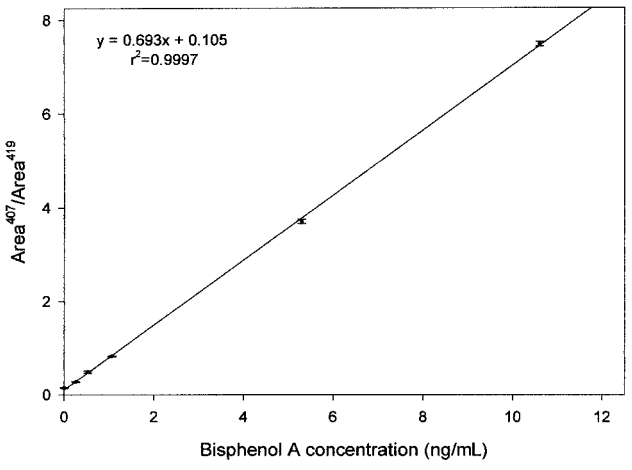

Figure 3. Calibration curves for the GC/MS. The amount injected onto the column can be calculated by multiplying the amount on this graph by the injection volume of 0.02 ml.

masses represent the fragment ions after the loss of $C_7F_5H_2$ from the disubstituted ether derivatives (Figure 1). The retention time of the derivatized BPA peak was 26.4 min.

Validation of this method involved analyzing duplicate samples on both the GC/MS and on the more selective GC/HRMS (Finnigan Mat 900, San Jose, CA). The conditions for the GC/HRMS were as follows: source temperature was 230°C, the reagent gas was isobutane, the transfer line temperature was 270°C, the electron energy was 130 eV, the emission current was 0.18 mA. The GC column was a DB-5, injector temperature was 250°C, the injection volume was 2 μl and the purge time was 1 min. The initial GC oven temperature was 75°C for 1 min then ramped to 200°C at 15°C/min then to 220°C at 10°C/min and then to 270°C at 15°C/min and held for 12 min. The total run time was 27 min.

Results

The product of the derivatization and the compound separated on the GC was the pentafluorobenzyl diether of BPA (Figures 1 and 2). The presence of a peak at 588 amu in the positive electron ionization (EI+) mass spectrum confirms the identity of the separated compound (Figure 1). In negative ionization mode, the predominant ion is formed by the loss of one of the pentafluorobenzyl moieties (Figure 1). The native and labeled BPA peaks elute at 26.4 min and are well resolved from surrounding peaks (Figure 2).

Calibration curves for BPA were linear from 0.26 to 10.6 ng/ml for the GC/MS and the GC/HRMS based on subtracting out a low background level (0.06 ng/ml urine; Figure 3). The detection limit for the GC/MS was 0.12 ng/ml (N=11) and for the GC/HRMS was 0.11 ng/ml (N=7). These detection limits were calculated by three standard deviations of the mean blank level of: 0.110±0.3 ng/ml (GC/MS) and 0.077±0.04 ng/ml (GC/HRMS). The method reproducibility at a sample concentration of 1 ng/ml was ±6% at 95% confidence(2σ). Constant percentage recovery (101.6±8.1%) and linear correlation were found

Table 1. Comparison of BPA levels in spiked human urine (ng/ml).

Spike amount	GC/MS		GC/HRMS	
(ng/ml)	Average*	S.D.	Average*	S.D.
0.27	0.25	0.04	0.27	0.02
0.53	0.57	0.07	0.57	0.04
1.06	1.06	0.02	1.02	0.02
5.30	5.43	0.06	5.36	0.16
10.6	10.9	0.14	10.9	0.26

*Background BPA levels in the unspiked urine were subtracted out of the spiked sample levels to allow easier comparison (N=3; GC/MS: 0.110±0.03; GC/HRMS:0.077±0.04). The unspiked urine pool is identical to Pool E in Table 2.

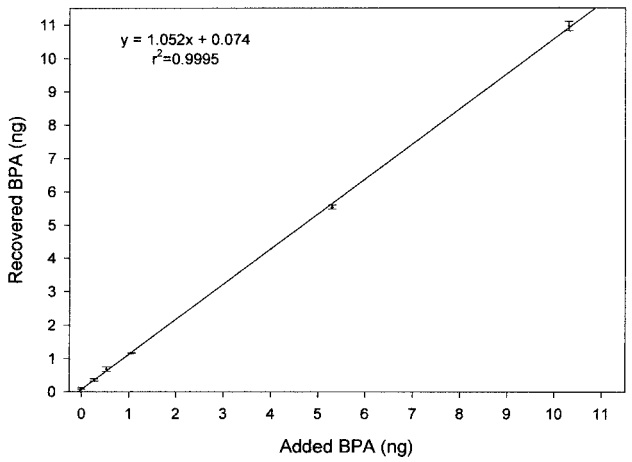

Figure 4. Standard addition and recovery in human urine.

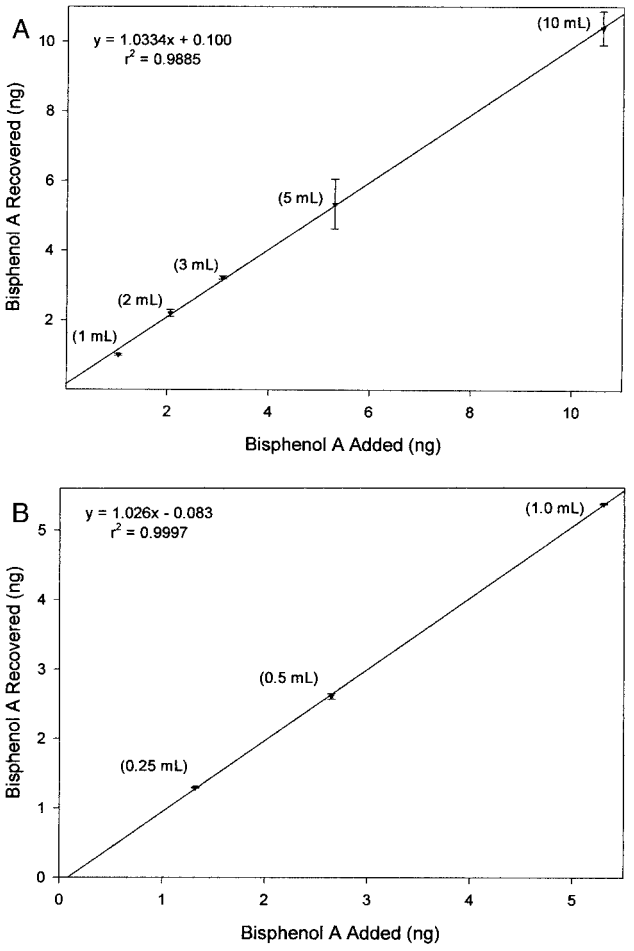

Figure 5. Effect of changing urine volume. (A) High volume and (B) low volume.

Table 2. Free and total BPA levels (ng/ml) in unspiked human urine*.

Pool	Free BPA	Total BPA
A	<0.12	0.51
B	<0.12	0.13
C	<0.12	0.16
D	<0.12	0.48
E	<0.12	0.11

*Urine specimens were collected anonymously and then pooled from at least five persons.

upon analysis of samples spiked with 0.25 to 10 ng BPA/ml (Figure 4). This relation shows that percentage recovery does not vary with concentration. As desired, the amount of BPA recovered varies linearly with the amount added.

This method was validated by analysis of duplicate samples by both GC/MS and GC/HRMS. The results obtained on duplicate samples analyzed by both instruments were not significantly different (Table 1). This agreement suggests that the GC/MS does not suffer from any major interferences that could inflate the values.

To improve the sensitivity of this method, different volumes of spiked urine samples were analyzed (Figure 5A and B). Sample volumes between 0.25 and 10 ml can be used with comparable results but larger sample volume may allow lower detection limits.

Pooled human urine samples analyzed by this method demonstrated no detectable BPA before deglucuronidation. After deglucuronidation, pooled urinary BPA levels varied from 0.11 to 0.51 ng/ml urine (Table 2).

Discussion

We have developed a highly sensitive and highly selective technique that should allow determination of background BPA levels in nonoccupationally exposed humans. The method involved SPE, derivatization with pentafluorobenzyl bromide and GC/MS. An alternative approach, the derivatization of BPA to trimethylsilyl (TMS) derivatives followed by GC/MS with EI+ was also attempted (data not shown). Although the TMS technique produced about 10-fold greater sensitivity when analyzing standards in water, the PFB technique described in this paper produced fewer interfering peaks when analyzing human urine or serum (data not shown). Other studies involving less complex matrices may be well served by using the TMS approach.

This method was developed for use in studies of human exposure to BPA and correlation with possible health effects. In rats, BPA produces a variety of hormone-like effects (Laws et al., 2000; Long et al., 2000). The health effects of BPA exposure on humans have not been studied.

This method's use of urine permits the inclusion of children in studies without requiring venous puncture. The method also offers flexibility by allowing use of different urine volumes. Studies with stored urine specimens often have different volumes available for analysis.

By omitting the enzymatic deglucuronidation step, we were able to measure free BPA in urine and serum. Preliminary data suggests that BPA in human serum is not present as the glucuronide but is present as the free phenol (Yoshimura et al., 2000). However, in our pilot work developing this method, BPA glucuronide was found in urine with very little free phenol (Table 2).

We will use this method to assess the BPA levels in human urine samples to provide a reference range. In addition, variations in human metabolism will also be examined by monitoring both free and glucuronidated BPA in urine and serum. Further, this method should allow easy expansion to related compounds like octyl and nonyl phenol as well as tetrabromobisphenol A and bisphenol F.

Acknowledgments

This work was supported in part by a grant-in-aid from the Ministry of Health and Welfare of Japan. Brand names are given for reference and do not constitute endorsement.

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