

Bisphenol-A: quantification in the urine of pregnant women by gas chromatography-mass spectrometry

Gabriel Guillermo Cases^{1a*}, Raul Eduardo Uicich¹, Paula Kandel Gambarte², Marcela Liliam Vázquez³, Claudio Fernando Moix³, Analía Ferloni^{4b}, Flavia Alejandra Vidal^{4b}, Lucas Otaño^{4b}, Silvana Figar^{4b}, Soledad Aragoné^{4b}, María Isabel Giménez^{1b}

Abstract

The use of bisphenol-A (BPA) in global industry has been increasing in recent years, with emerging markets driving this growing demand. BPA applications in the food and beverage industry account for only 3 to 4% of global polycarbonate consumption, but its use is being re-examined as several scientific papers have come to light indicating a direct link between BPA and adverse health effects. Contamination of food and beverages occurs by migration of BPA from the containers that contain it (canned foods, wines, etc.), and is the main source of human exposure. To evaluate such exposure, an analytical method by gas chromatography coupled to mass spectrometry was developed and validated for the quantification of total BPA in urine of pregnant women attended at the Italian Hospital of Buenos Aires in 2013, with a limit of quantification of 2.0 ng/mL and a limit of detection of 0.8 ng/mL. Of the 149 urine samples analyzed, 66.4% were quantifiable, with the median total BPA of 4.8 ng/mL (4.3 ng/mg creatinine) and the geometric mean of 4.8 ng/mL (4.7 ng/mg creatinine).

Keywords: Bisphenol-A; Urine; Pregnancy; Endocrine disruptor; Chromatography gas chromatography-mass spectrometry

¹ Biochemist.

² Degree in Biological Sciences.

³ Chemical Technician.

⁴ Physician.

^a Principal Professional. CPA. CONICET.

^b Member of the Health and Environment Research Program (PISA).

Chromatography and Mass Spectrometry Section, Central Laboratory, Hospital Italiano de Buenos Aires, Perón 4190, C1181ACH, Ciudad Autónoma de Buenos Aires, Argentina.

* Author for correspondence.

Introduction

Bisphenol-A (bisphenol-A; BPA) is an organic compound from which polycarbonate, a plastic widely used in the manufacture of food and beverage containers, is produced. Epoxy resins are also mostly made from BPA and are used as a coating for food and beverage containers (cans, wine storage tanks, etc.), forming a film between the food and the inner surface of the metal container, which prevents corrosion and prevents migration of metal into the food. But what cannot be avoided is the migration of BPA, however minimal, from the coating into the food or beverage. Therefore, people are exposed to small amounts of BPA just by eating. Several scientific studies have assigned BPA a biologically active role at very low doses (1), as it has the ability to bind to estrogen receptors (endocrine disruptor). These studies indicate a close correlation between BPA and harmful effects on human health.

Consequently, its use in the food industry is being re-examined, driven mainly by public opinion, the media and environmental activists. Both the European Food Safety Authority as well as the US Public Health Service are reviewing all the data collected in recent years on BPA (2) (3). For this purpose, a period of one to two years has been set during which they will not take extreme corrective measures, but will adopt a more lax stance, in order to give the food packaging materials industry time to develop alternative proposals to BPA that will help to solve the problem.

BPA is rapidly absorbed in the gastrointestinal tract and in a high proportion after oral intake at low doses comparable to average human environmental exposure. It undergoes virtually no phase I biotransformation and is metabolized in the liver almost completely by conjugation in a

phase II biotransformation with glucuronide (in higher proportion) and sulfate, which add hydrophilicity to BPA and allow its rapid urinary excretion.

Efficient hepatic conjugation of BPA releases very low levels of free BPA (BPA-L) capable of binding to the estrogen receptor into the plasma compartment. Therefore, the formation of BPA-glucuronide (BPA-G) and BPA-sulfate (BPA-S) are considered as deactivation or detoxification reactions, although recent scientific work indicates that BPA-G may also be biologically active (4) (5). In urine there are very low levels of BPA-L and the main metabolite present is BPA-G, so urine is considered the body fluid of choice for assessing human exposure to BPA through the quantification of the most appropriate biomarker of exposure, total BPA (BPA-T) (6). To achieve reliable and robust results, analytical methods based on mass spectrometry are the most suitable because of their high selectivity, sensitivity and precision (4) (6-10). The objective of the present work is the development of an analytical method by gas chromatography coupled to a mass spectrometry detector (GC/MS) for the detection and quantification of BPA-T in urine of pregnant women, from a procedure that includes: enzymatic hydrolysis, extraction with organic solvent of the compound and its subsequent derivatization.

Materials and Methods

Materials

Bisphenol-A standard (99%), beta-glucuronidase/sulfatase enzyme (Type H-1 from *Helix pomatia*) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) derivatizing agent were purchased from Sigma-Aldrich (Argentina). Ethyl acetate

(LC/MS grade) was Carlo Erba brand, ammonium acetate from Fluka and hydrochloric acid from Merck. The Sartorius Arium Pro ultrapure water system and 150 mL urine collection bottles, phthalate-free and BPA-free, certified by Deltalab Laboratory, were used. The glassware was new and rinsed with ultrapure water.

Preparation of Calibration Standards

The BPA standard was dissolved in ethyl acetate and a 1.1 mg/mL stock solution was prepared. Working solutions were prepared by serial dilutions in ethyl acetate of the BPA stock solution. These were used to perform the calibration curve for five concentration levels, ranging from 2.0 to 20.0 ng/mL. The stock solutions were stored at 4 °C. The working solutions were stored at 4 °C and protected from light for no more than one week.

Sample collection and preparation

Urine samples by spontaneous urination were collected in the hospital setting in a polypropylene bottle (BPA-free), with at least three hours of retention and without prior fasting, then aliquoted in polypropylene tubes and stored in a freezer at -80 °C until analysis in the laboratory. A total of 149 urine samples from pregnant women (11 to 16 weeks), of legal age, attended at the Italian Hospital between August and September 2013 were analyzed. The objectives of the study were explained to them, confidentiality was guaranteed and their written informed consents were obtained. The samples were collected with the approval of the Ethics Committee for Research Protocols of the Hospital Italiano de Buenos Aires (CEPI, Protocol No. 1592).

Sample processing

For BPA quantification, a procedure

described previously (7) (8) (10) was used with slight modifications. Urine samples with a creatinine concentration between 25 mg/dL and 360 mg/dL were selected. 1 mL of urine was placed in a glass tube, 50 µL of 1 M ammonium acetate buffer solution (pH: 5.0) and 50 µL of -glucuronidase were added and incubated for 24 hours in a thermostated water bath at 37 °C. Then 150 µL of 4 N hydrochloric acid and 2 mL of ethyl acetate were added to perform the extraction of BPA-T (BPA-L+BPA-G). To facilitate the extraction, the sample was shaken in a vortex for 4 minutes, then centrifuged at 2,500 rpm and at 4 °C for 5 minutes. A supernatant was obtained which was transferred to a glass vial and evaporated to dryness under nitrogen stream. For derivatization, 100 µL of the BSTFA + 1% TMCS (silylating reagent) mixture was added to the dried extract and incubated for 30 min at 60 °C in a heating block (dry heat). 2 µL of the derivatized sample was injected into the GC/MS through the automatic injector.

Creatinine determination was performed by the Jaffé method in a BeckmanCoulter AU5800 autoanalyzer.

Separation and quantification

All BPA analyses were performed using an Agilent Technologies model 7890A gas chromatography equipment coupled to a mass selective detector of the same brand, model 5975C TAD (GC/ MSD), and an autosampler model 7693. The Agilent GC/MSD ChemStation instrument control program was used for data acquisition and spectra processing. An Agilent J &W DB-5MS + DuraGuard capillary column (30 m x 0.250 mm ID, 0.25 µm film thickness) was used for chromatographic separation. The column temperature was programmed as follows: the initial temperature was 150 °C for 2 minutes

and was increased at 30 °C/minute up to 270 °C, this final temperature was maintained for 8 minutes. It was rinsed (back flush) at 300 °C for 8 minutes. A solvent delay of 3 minutes was applied before the start of the mass scan to protect the ion multiplier of the detector from saturation. The total run time was 14 minutes. High purity helium was used as carrier gas, at a constant flow rate of 1 mL/min. The injector temperature was set at 250 °C, and the injection volume was 2 µL in splitless mode. The temperatures of both the GC/MSD interface and the ion source were 300 °C, and the quadrupole temperature was 180 °C. The electron impact ionization energy (EI) was 70 eV. Ion acquisition was performed by selective monitoring mode (SIM). The ions monitored were m/z 357 for quantification, corresponding to demethylated bis(trimethylsilyl)-BPA (M-15)⁺, and m/z 358, 360 and 372 for confirmation, the latter corresponding to bis(trimethylsilyl)-BPA (M⁺ ion) (Fig. 1). For the identification and confirmation of BPA in the samples, the retention time (Tr) and the ratio of peak areas of the confirmation ions with respect to the quantification ion,

parameters obtained from the BPA standard, were used. As additional identification data, the BPA spectrum of the sample was searched in the commercial NIST library (Fig. 2). The concentration of BPA in each sample was measured by interpolation of the peak area of the analyte in the calibration curve.

Assessment of GAP contamination

Various blanks of all materials used from sample collection, storage and subsequent analysis were analyzed. Sample vials, glass tubes, pipette tips, autosampler vials, solvents and reagents, etc. were evaluated. In addition, the contribution of BPA from the GC/MSD system was analyzed. The presence of BPA above the detection limit (LOD) was not detected in any of the tests performed.

Method validation

A procedure based on the validation criteria published by the EMA (11) was used. The GC/MS method was validated for selectivity, linearity, limit of detection (LOD), lower limit of quantification (LQoL), accuracy and precision.

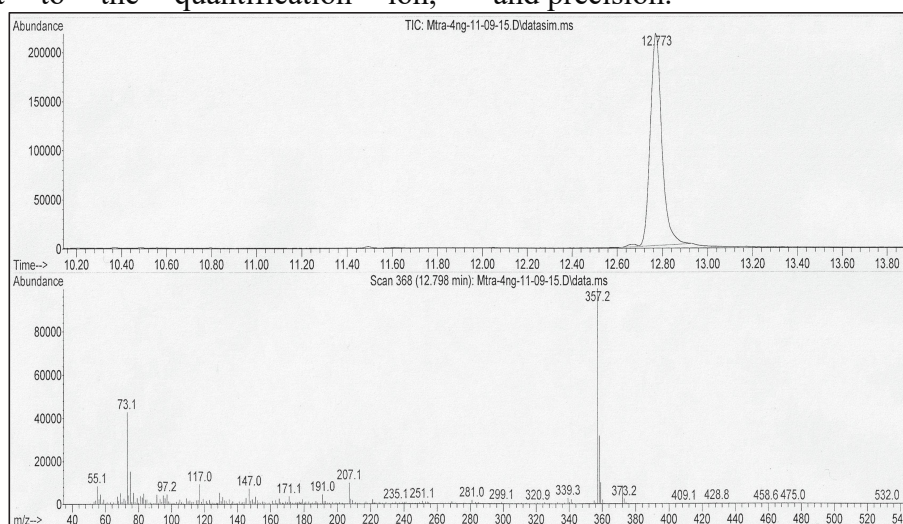


Figure 1. SIM chromatogram and mass spectrum of BPA.

Selectivity was evaluated by comparison of the chromatographic peak area between the

sample blank and the calibration standard sample. The peak area at the expected retention

time for each analyte in the blank samples should be less than 20% of the average peak areas in the LIC samples.

The calibration curve was constructed by plotting the peak area of the measured analyte on the ordinate axis (Y axis) against the nominal concentration of the analyte on the

abscissa axis (X axis), for each of the calibrators.

A linear least squares regression analysis was performed, and the slope, the ordinate to the origin and the coefficient of determination (R²) of the calibration curve were calculated.

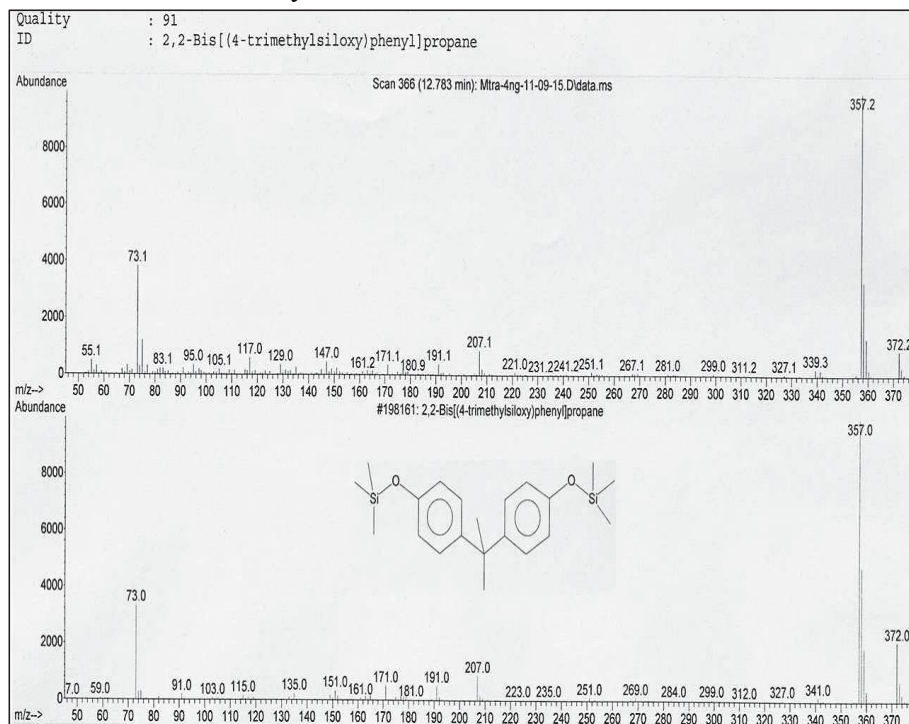


Figure 2. BPA: search and comparison with library spectrum.

The LIC of the assay was defined as the lowest concentration of the calibration curve that could be quantified (LIC, signal-to-noise ratio (S/N)≥5). The LOD was defined as the lowest concentration that could be detected (LOD, S/N≥3).

Data analysis and statistics

ChemStation software was used to generate the calibration curve containing: retention time (Tr), signal area values, and concentration for the analyte in each sample. Calculations of the linear relationship between signal areas and concentrations were obtained by least squares regression with a weighting factor of 1/x².

The Microsoft Office Excel program was used for data analysis. The mean with its standard deviation (SD), geometric mean (GM), geometric standard deviation (SD) and median were calculated for creatinine-adjusted and non-creatinine-adjusted T-PAP.

Results

Selectivity

The analytical method made it possible to differentiate BPA from other urine components.

Linearity, detection and quantification limits

The calibration curve was found to be linear between 2.0 and 20.0 ng/mL, with a

coefficient of determination (R^2) greater than 0.99. The limit of detection was 0.8 ng/mL and the limit of quantification was 2.0 ng/mL (both limits were analyzed for BPA in 1 mL of urine). All BPA concentrations in each sample were adjusted for the concentration of creatinine in the samples and expressed as ng/mg creatinine to control for variable urine dilutions.

Precision and accuracy

These parameters were evaluated on a quality control sample of 10 ng/mL, by quintuplicate preparation and injected by simplified in the same run. The following values were obtained: precision: 12.8% (expressed as percentage coefficient of variation CV%), accuracy: 10.3% (expressed as percentage relative error ER%).

Total BPA values found in urine

The presence of BPA-T was detected in 87.2% (130/149) of the total samples analyzed. The mean obtained from the total number of samples quantified was 5.7 ng/mL (standard deviation: 4.3 ng/mL), and 5.7 ng/mg (SD: 5.0 ng/mg) (creatinine-adjusted values). Only three samples were above the upper limit of

quantification (ULQ). Table I summarizes the values obtained with the validated method.

Discussion and Conclusions

An analytical method of high sensitivity and specificity was used for the detection and quantification of trace amounts of BPA-T present in the urine of pregnant women in their first trimester of gestation. The main metabolite of BPA in urine is BPA-G (about 70% of BPA-T), followed in proportion by BPA-S (15%) and the rest is unconjugated BPA (BPA-L) (4). To release the conjugated BPA and obtain BPA-L, the enzyme beta-glucuronidase was used, which in addition to hydrolyzing BPA-G has a sulfatase action. Although the pH used in the hydrolysis was optimal for glucuronidase action, it is possible that some of the BPA-S was hydrolyzed. Therefore, the BPA-L obtained after enzymatic hydrolysis would represent the total BPA that entered the body and should be considered as the only sufficiently reliable biomarker of exposure in population-based studies (6). Furthermore, in the particular case of pregnant women, it would serve as an indirect marker of fetal exposure.

Table I. Summary of the results obtained for total BPA in urine of pregnant women.

| Total GAP | BPA value (ng/mL) | Number of samples (n) | of total |
|--------------------|-------------------|-----------------------|----------|
| <LD | Less than 0.8 | 19 | 12,8 |
| \geq LD and <LIC | From 0.8 to 2.0 | 31 | 20,8 |
| Quantifiable | From 2.0 to 20.0 | 99 | 66,4 |

The presence of BPA was detected in 87.2% of the urine samples analyzed. This shows the omnipresence of this compound in daily life and the difficulty of finding samples that serve as a non-exposure control for the comparison of results. In strictly analytical terms, measures should be taken to minimize BPA contamination in the materials used

throughout the process, from sample collection to quantitative analysis.

A median of 4.3 ng/mg creatinine and 4.8 ng/mL (unadjusted) was obtained for the concentration of T-BPA. The MG (adjusted for creatinine) was 4.7 ng/mg, and 4.8 ng/mL (without adjustment), and the SDR for both was 1.7 (Table II).

Creatinine concentrations were also used to determine whether the spontaneous urinary voiding sample was valid.

Table III compares the values obtained in this study with those published in the literature. For the most part, there is a high percentage of BPA detection (greater than 80%) in the urine samples of pregnant women. Regarding the GM and median values, the authors believe that their comparison with other works is inadequate, due to the variability that exists between the sample processing techniques (the use or not of enzymatic

hydrolysis, derivatization, etc.), in chromatographic analysis (liquid or gas chromatography) and at the different times of gestation in which the samples were collected. In addition, there is scientific evidence that shows a low correlation between serial measurements of urinary BPA of the same person during pregnancy (12). This is due to the nature of BPA exposure (episodic food consumption and product use), and its short biological half-life (13). Therefore, estimating long-term exposure to BPA using only a spot urine sample is imprecise (14).

Table II. Urinary total BPA values in pregnant women (less than 12 weeks gestation).

| Total GAP | MG (SDR) | Median | Mean (SD) |
|---------------------------------|-----------|--------|-----------|
| Adjusted for creatinine (ng/mg) | 4,7 (1,7) | 4,3 | 5,7 (4,3) |
| Not adjusted (ng/mL) | 4,8 (1,7) | 4,8 | 5,7 (5,0) |

Table III. Published values of total BPA in urine of pregnant women (2013-2019).

| Study | Year | City-Country | Sampling | n ^a | %≥LD ^b | Method | LD (ng/mL) | Total BPA (ng/mL) | |
|-----------------------|------|-------------------------|---------------------------------|----------------|-------------------|----------|------------|-------------------|--------|
| | | | | | | | | MG ^c | Median |
| Italian Hospital | 2019 | Buenos Aires Argentina | <12 weeks | 149 | 87,2 | GC/MS | 0,80 | 4,8 | 4,8 |
| Giesbrecht et al (15) | 2017 | Alberta-Canada | <27 weeks | 132 | 90,9 | LC/MS | 0,32 | 1,07 | 0,89 |
| Chiu et al (16) | 2017 | Boston-USA | 1st quarter | 208 | 83 | LC/MS | 0,4 | 1,15 | NR |
| Johns et al (17) | 2017 | Boston-USA | 10 weeks | 476 | 82 | LC/MS | 0,4 | NR ^c | 1,28 |
| Gerona et al (4) | 2016 | San Francisco USA | 2nd quarter | 112 | 100 | LC-MS | 0,05 | 7,69 | 4,61 |
| Huo et al (18) | 2015 | Wuhan-China | Within 3 days prior to delivery | 452 | 89,4 | UPLC-MS | 0,2 | 2,42 | 2,60 |
| Myridakis et al (19) | 2015 | Crete-Greece | 10-13 weeks | 239 | 99,6 | LC/MS | 0,01 | 1,2 | 1,2 |
| Arbuckle et al (20) | 2014 | 10 cities in Canada | <14 weeks | 1936 | 87,7 | GC/MS-MS | 0,2 | 0,80 | 0,82 |
| Lee et al (21) | 2014 | 3 cities in South Korea | 3rd quarter | 757 | NR | LC/MS | 0,28 | 1,29 | 1,08 |
| Hoepner et al | 2013 | New York USA | 3rd quarter | 375 | 94 | LC/MS | 0,4 | 1,8 | 1,8 |

| | | | | | | | | | |
|---------------------|------|-----------------|-------------|-----|------|-------|-----|-----|-----|
| (22) | | | | | | | | | |
| Casas et al (12) | 2013 | Catalonia-Spain | 3rd quarter | 479 | 99,4 | LC/MS | 0,1 | 1,8 | 1,8 |

^an: number of samples. ^bLD: limit of detection. ^cMG: geometric mean. ^eNR: not reported.

The results of this work are the first data obtained on urinary BPA-T in a population of pregnant women in Argentina, and should be taken as the starting point for future toxicological and environmental assays that evaluate long-term exposure to BPA, and at very low concentrations, both in newborn children and in the general population of Argentina.

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Conflicts of interest

The authors declare that they have no conflicts of interest with respect to this work.

Correspondence

Dr. GABRIEL CASES
 Perón 4190, Ground Floor
 (C1181ACH) CIUDAD AUTÓNOMA
 DE BUENOS AIRES,
 Argentina
 Tel. (54) (11) 4959-0200 ext. 4900 /
 Tel. (54) (11) (54) 15-4039-2791
 E-mail:
 gabriel.cases@hospitalitaliano.org.ar;
 ggcases@hotmail.com

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