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Direct LC-MS/MS and indirect GC–MS/MS methods for measuring urinary bisphenol A concentrations are comparable

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Abstract

Background: Bisphenol A (BPA) is typically measured in urine using an indirect method that involves enzymatic deconjugation and extraction. In contrast, the direct method measures free and conjugated BPA concurrently and sums them to estimate urinary BPA concentrations. Statistical comparison of total BPA results using the direct and indirect methods is necessary to accurately interpret biomonitoring data for risk assessments.

Objectives: To compare urinary BPA concentrations estimated from the indirect and direct methods in duplicate first trimester urine samples collected from 1879 pregnant women from the MIREC Study.

Methods: For the indirect method, we measured urinary BPA concentrations using GC–MS/MS. For the direct method, we summed free and conjugated BPA concentrations measured using LC-MS/MS. We evaluated deviation between the two methods using the Bland-Altman analysis in the total sample and stratified (1) by specific gravity and (2) at the limit of quantification (LOQ).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106874.

Results: Median urinary BPA concentrations for the direct and indirect methods were 0.89 μg BPA equivalents/L and 0.81 μg/L respectively. Concentrations from the direct method were, on average, 8.6% (95% CI: 6.7%, 10.5%) higher than the indirect method in a Bland-Altman analysis. The percent differences between the two methods was 4.0% in urines with specific gravities < 1.02 (n = 1348, 72%) and 20.3% in urine with specific gravity ≥ 1.02. In values below the LOQ (n = 663, 35%), we observed smaller average percent deviation (4.8%) between the two methods but wider limits of agreement.

Discussion: Results from this study, based on the largest statistically rigorous comparison of the direct and indirect methods of BPA measurement, contrast previous findings reporting that the indirect method underestimates total BPA exposure. The difference in urinary BPA concentrations we observed with the indirect and direct methods is unlikely to alter the interpretation of health outcome data.

Keywords

Bisphenol A; Urine; Pregnancy; Biomonitoring; Phenols; BPA glucuronide; Environmental pollutants

1. Introduction

Bisphenol A (BPA) is a high production volume chemical that is widely used in the manufacture of consumer products including polyvinyl chloride, polycarbonate plastics, epoxy resins of aluminum cans, and in thermal receipt paper (CDC, 2017). BPA enters the environment during the production, processing, use, and disposal of BPA-containing products (Environment and Climate Change Canada, 2008).

Although BPA exhibits low potential for bioaccumulation (Environment and Climate Change Canada, 2008) and has an estimated half-life of less than 6 h in humans (Teeguarden et al., 2015; Thayer et al., 2015), its ubiquitous presence in the environment leads to widespread and repeated exposure. BPA has been detected in over 90% of study participants in national biomonitoring studies (Calafat et al., 2008; Health Canada, 2019; Covaci et al., 2015; Tschersich et al., 2021) and pregnancy cohorts (Braun et al., 2011; Cantonwine et al., 2010; Casas et al., 2013; Harley et al., 2013; Snijder et al., 2013). Ingestion of contaminated food is the primary route of exposure (Christensen et al., 2012), but dermal absorption and inhalation are also possible (Biedermann et al., 2010; Dekant and Völkel, 2008; Kang et al., 2006; Zalko et al., 2011). Frequent contact with thermal receipt paper, for example, is recognized as a source of BPA exposure (Ndaw et al., 2016; Thayer et al., 2016).

Once ingested, BPA is metabolized by the liver and almost completely undergoes phase II conjugation via glucuronidation resulting in water soluble, hydrophilic metabolites. These conjugated metabolites, which lack estrogenic activity (Völkel et al., 2002), are excreted in urine (Koch et al., 2012). Total BPA has been traditionally measured using an indirect approach that converts the conjugated metabolites back to free BPA using enzymatic hydrolysis (Andra et al., 2016; Lakind et al., 2012). Recent developments in synthesizing BPA conjugates and internal standards has facilitated direct measurement of free BPA and its conjugates using liquid chromatography coupled with mass spectrometry (Andra et al.,

2016; Liao and Kannan, 2012; Provencher et al., 2014). BPA conjugates, including BPA disulfate (BPADS), BPA glucuronide (BPAG), and BPA monosultate (BPAS), as well as free BPA identified from this direct approach can then be summed to create an estimate of total BPA.

Authors of a review on recent advances in BPA analytical methodologies have noted the need for comparison of BPA concentrations using the frequently named "indirect" GC–MS/MS and "direct" LC-MS/MS methods (Andra et al., 2016). Studies comparing the two methods are scarce, limited by sample size and lack of rigorous statistical methods for examining agreement between the two methods. In their comparison of the two methods, Gerona et al (2020) concluded that the indirect method underestimates exposure by an order of magnitude or more, particularly notable at the highest concentrations. Furthermore, these authors reported that their geometric mean from the direct method results were 44 times higher than the geometric mean BPA concentrations for adults in the latest cycle of the US National Health and Nutrition Examination Survey (NHANES) (measured as total BPA after enzymatic hydrolysis) (Gerona et al., 2020). This study was based on 29 pregnant women and the authors did not conduct any statistical analysis to compare agreement between the two methodological approaches used by the authors. Another comparison of the two methods based on samples from 46 participants reported a strong correlation (Spearman r = 0.86) between the GC–MS/MS and LC-MS/MS methods and 12.6% higher measurements in the direct method than the indirect method (Provencher et al., 2014). Comparison of descriptive statistics and calculation of correlation coefficients do not provide an indication of agreement between the two laboratory methods (Bland and Altman, 2003). Relying on descriptive statistics and correlation, therefore, hinders the ability to address the critical question of whether the traditional indirect method of quantifying total and free BPA after hydrolyses is consistent with results from the more recently applied direct method of measuring free and conjugated BPA conjugates separately and summing them to calculate total BPA. Considering the developmental toxicity of BPA and the vulnerability of the developing fetus to exogenous chemicals (NTP, 2008), accurate exposure assessment during pregnancy is critically important for understanding exposure, interpreting health outcome data, and informing risk assessment.

The purpose of this study was to conduct a formal statistical assessment of agreement between total urinary BPA concentrations measured by the indirect and direct method in a pan-Canadian cohort of pregnant women. Our analysis capitalized on the largest national-level dataset that contained total BPA results produced from the direct (LC-MS/MS) (Arbuckle et al., 2015) and the indirect (GC–MS/MS) (Arbuckle et al., 2014) analysis of the same first trimester urine samples.

2. Methods

2.1. Study population

The Maternal-Infant Research on Environmental Chemicals (MIREC) study is a trans-Canadian pregnancy cohort of 1983 women recruited in their first trimester of pregnancy from 10 sites across Canada between 2008 and 2011 (Arbuckle et al., 2013). Eligibility criteria included ability to consent and communicate in English or French, age 18 or over

at the time of recruitment, planning on delivering at a local hospital, and willing to provide a cord blood sample. Women consented to provide biospecimens and detailed clinical data throughout pregnancy. The research ethic boards at Health Canada and St. Justine's Hospital (Montreal, QC), as well as all recruitment sites approved the study protocol, and women provided informed consent to participate. For the purposes of the present analyses, we restricted the population to women with BPA measurement data from both the indirect (GC–MS/MS) and direct (LC-MS/MS) methods (n = 1879).

As previously described, we collected spot urine samples between 6.1 and 14.9 weeks gestation (mean 12.4) (Arbuckle et al., 2014). Urine was collected in polypropylene cups, aliquoted into 30-mL Nalgene® tubes, frozen at –20 °C, and shipped on dry ice to the MIREC Biobank. We briefly summarize the two methods employed to measure total, free and conjugated BPA. All laboratory analyses were performed at the Centre de Toxicologie du Québec (CTQ), Institut national de santé publique du Québec (INSPQ). This laboratory is accredited by the Standards Council of Canada under ISO 17025, the international standard for technical competence and quality in all areas of testing and calibration.

2.2. *Total BPA using indirect GC–MS/MS method*

As previously described (Arbuckle et al., 2014; Provencher et al., 2014), a 3-step process of enzymatic hydrolysis, derivatization and extraction was used to free the conjugated compounds in urine to measure total BPA. Urine samples of 100 µL were spiked with BPA-$^{13}C_{12}$ and deconjugated with *Helix pomatia* b-glucuronidase enzyme (type HP-2) for 3 h at 37 °C and pH 5.0, prior to direct derivation with pentafluorobenzyl bromide. Pentafluorinated benzyl derivatives were then extracted with a mixture of hexane and dichloromethane and analyzed by GC–MS/MS with a GC Agilent 6890 N (Agilent Technologies; Mississauga, Ontario, Canada) coupled with a tandem mass spectrometer Quattro Micro GC (Waters; Milford, Massachusetts, USA). The measurement of ions generated was performed in multiple reaction monitoring (MRM) mode with a source in negative chemical ionization mode (NCI). The analytical column used was a HP-5MS 30 m × 0.25 mm × 0.25 µm film thickness (Agilent Technologies; Mississauga, Ontario, Canada).

2.3. Sum BPA using direct LC-MS/MS method

Details of this methodology were provided in prior reports (Arbuckle et al., 2015; Provencher et al., 2014). Briefly, free BPA and its isotope-labeled standard BPA-$^{13}C_{12}$ were derivatized with dansyl chloride directly in 1 mL of urine. A liquid–liquid extraction with hexane was subsequently performed and the organic phase evaporated prior to reconstitution in a solution of acetonitrile: H_2O (50:50). The LC-MS/MS (UPLC Acquity and Xevo TQ-S;Waters; Milford, Massachusetts, USA) was operated in electrospray positive and MRM mode. Chromatographic separation was achieved on an Acquity UPLC HSS T3, 1.8 µm, 50 × 2.1 mm analytical column (Waters; Milford, Massachusetts, USA) using a mobile phase gradient with 0.1% aqueous formic acid solution and acetonitrile. Contamination was minimized by derivatization with dansyl chloride at the beginning of the process; this derivatization was also used to increase the sensitivity of the method for free BPA. The conjugated metabolites BPAS, BPADS, BPAG, and their isotope labeled standards BPAS-d_6, BPADS-d_6, BPAG-d_6, were extracted from 1.5 mL of urine by solid phase extraction using

a weak anion exchange phase (Strata X-AW; Phenomenex; Torrance, California, USA). Analytes were eluted from the cartridge using a solution of 1% ammonium hydroxide (NH_4OH) in methanol. The extracts were evaporated to dryness and reconstituted in a solution of 25% methanol in water. The same LC-MS/MS instrument and analytical column were used as for the free species, but the MS/MS was operated in the electrospray-negative and MRM mode. A mobile phase gradient from aqueous NH_4OH (2%) pH 11.0 to an NH_4OH–methanol solution (0.1%) was used to obtain proper chromatographic resolution of conjugated compounds. The concentrations for all the conjugated forms were expressed in BPA equivalents so they could be summed. The limits of quantification (LOQ) and detection (LOD) for both methods are detailed in Table 1.

2.4. Quality control and quality assurance

The laboratory undertook a number of measures to minimize potential contamination and sources of error. We adjusted the pH of the mobile phase to 11.0 and used an appropriate gradient chromatography to ensure separation of the conjugate and compounds with similar molecular weight (i.e. resveratrol glucuronide). Quality control samples, reagent blanks, and urine blanks were incorporated into each batch of samples. Reference materials for the BPA conjugates was obtained by spiking urine samples with three different concentrations. Potential contamination from collection and storage material was assessed using field blanks (Arbuckle et al., 2015). Last, our INSPQ laboratory participates in OSEQAS, an external quality assessment scheme for organic substances in urine, and spikes proficiency test materials with BPAG at specific gravities between 1.013 and 1.020.

2.5. Statistical Analysis

We calculated descriptive statistics and Spearman correlation coefficients for the indirect GC–MS/MS and direct LC-MS/MS methods for women who had data available for both methods. The indirect GC–MS/MS method recorded values below the limit of detection (LOD) as < LOD. The direct LC-MS/MS method provided machine readings for the individual congeners as well as free BPA. To derive Sum BPA (direct LC-MS/MS method), we summed the concentrations of free and conjugated BPA and used the LOD of the most dominant conjugate (BPAG). To facilitate comparison of the direct LC-MS/MS and indirect GC–MS/MS measurements, we substituted values below the LOD with the LOD/2 and applied the GC–MS/MS method LOD of 0.2 μg/L to both methods. All results from the LC-MS/MS direct method are expressed as μg BPA equivalents/L. We did not standardize urine for hydration because we compared aliquots from the same urine sample collected from each woman.

To examine the agreement and visualize the differences between the direct LC-MS/MS and indirect GC–MS/MS methods, we used the Bland-Altman method (Bland and Altman, 2003). Using this approach, we plotted the average of the two methods (x-axis) against the percent differences (y-axis), calculated the mean percentage differences, and the 95th percentile limits of agreement. Under normally distributed data for the differences, 95% of the data will lie within these limits of agreement. The standard error of these limits is approximately $\sqrt{3s_D^2/n}$ where s_D is the standard deviation of the differences and n is the sample size. As the assumptions were not met for the unit differences, we calculated

the percent differences in order to meet the Bland-Altman assumptions of (i) normal distribution of the differences (y-axis values) and (ii) independence of the average (x-axis) and differences (y-axis) of two methods. The first assumption was verified by normality tests and plots and the independence assumption was verified by a null hypothesis test of a correlation coefficient r = 0 between the average (x-axis) and difference (y-axis) values. The percent difference for each woman's urine was calculated by the following formula:

$$(\text{Sum BPA} - \text{Total BPA})/((\text{Sum BPA} + \text{Total BPA})/2)$$

where the Sum BPA is the sum of free and conjugated BPA measured by the LC-MS/MS direct method and the Total BPA is measured by the GC–MS/MS indirect method. The percent difference was, therefore, calculated as the BPA unit difference divided by the average BPA value for each subject.

We conducted two sensitivity analyses to explore potential sources of discrepancies between the two methods due to (1) differing urinary specific gravity (SG) levels and (2) imprecision below the LOQ. We hypothesized that higher urinary specific gravities could result in incomplete deconjugation. The indirect GC–MS/MS method may underestimate Total BPA concentrations (Andra et al., 2016). To explore this hypothesis, we created Bland-Altman plots and calculated mean percentage differences between the two methods among the subgroup of individuals with specific gravity greater than or equal to 1.02 compared to less than 1.02. This threshold has been identified as an indicator of dehydration (Kavouras, 2002). The LOQ is the lowest analyte concentration that can be quantitatively detected with stated accuracy and precision lower than 25% (Taylor, 1987). To assess deviation between the two methods below vs above the LOQ, we calculated the average of the indirect GC–MS/MS and direct LC-MS/MS method for each sample and stratified according to the LOQ of the indirect method (0.8 μg/L). This approach allowed us to include all values rather than excluding values where results from one method were above the LOQ and results from the other method were below the LOQ. Furthermore, we used the LOQ of the indirect method because each of the conjugated metabolites have different LOQs according to the direct method.

All analyses were performed in SAS EG v. 7.1.

3. Results

Among the women enrolled in MIREC, 1879 women had measurements of BPA using both the indirect GC–MS/MS and direct LC-MS/MS method. When applying the LOD of 0.2 μg/L to both methods and substituting LOD/2 for non-detects in both methods, the median Sum BPA (direct LC-MS/MS method) and Total BPA (indirect GC–MS/MS method) concentrations were 0.89 μg BPA equivalents/L and 0.81 μg/L respectively. The percentage of non-detects for Sum and Total BPA was 10.8% and 12.1%, respectively (Table 1).

The Spearman correlation coefficient between the Sum (direct LC-MS/MS method) and Total BPA (indirect GC–MS/MS method) was 0.93 ($P < 0.0001$). The scatterplot with a

log-10 scale for both X and Y axes is depicted in Fig. 1; 61% of values were above the identity diagonal and higher in the direct LC-MS/MS method than the indirect GC–MS/MS method. The mean unit difference between the two methods was 0.20 μg/L (95% CI: 0.12, 0.29) with 95% limits of agreement ranging from −3.42 to 3.83. We interpret these results with caution however, because, as previously noted, the Bland-Altman assumptions for independence and normality were not met for unit differences.

Based on the Bland-Altman plots, the mean percent differences between the two methods was 8.6% (95% CI: 6.7%, 10.5%) with the direct LC-MS/MS method tending to produce higher, on average, concentrations than the indirect GC–MS/MS method (Table 2, Fig. 2). The lower and upper 95% limits of agreements for the percent differences were −72.6% (95% CI: −75.8%, −69.3%) and 89.8% (95% CI: 86.5%, 93.0%), respectively (Table 2). As demonstrated in Fig. 2B, the magnitude of the percent differences was highest at the lowest concentrations and for values where one method produced a value that was above the LOD and the other method produced a value that was below the LOD. These values are depicted by the symmetrical distinct arcs observed above and below the horizontal mean difference line.

When stratified by specific gravity, the mean percentage differences between the Total BPA (indirect GC–MS/MS method) and Sum BPA (direct LC-MS/MS method) was 20.3% (95% CI: 17.5%, 23.1%) for samples with specific gravities greater than or equal to 1.02. The mean percentage differences was 4.0% (95% CI: 1.7%, 6.3%) in samples with specific gravities lower than this threshold (Table 3). As shown in Fig. 3, the 95% limits of agreement are wider in the subgroup with specific gravity less than 1.02. Sixty-two percent of samples in this subgroup (specific gravity less than 1.02) were below the indirect GC–MS/MS method LOQ of 0.8 μg/L.

When stratified by the LOQ, we observed wider limits of agreement in the values below the LOQ than values above the LOQ (Fig. 4B) due to large percentage differences that arise when one value is above the LOD and the other is below the LOD. The mean percent difference (4.8%) was, however, lower in this subgroup (<LOQ) than in values above the LOQ (10.8%).

4. Discussion

We compared results of the direct LC-MS/MS method and indirect GC–MS/MS methods of measuring urinary BPA concentrations in the largest to date population (n = 1879) by applying formal statistical methods of assessing agreement. We observed that Sum BPA (direct LC-MS/MS method) results were highly correlated with and, on average, 8.6% higher than the Total BPA (indirect GC–MS/MS method) results using the Bland-Altman formula. Based on these results, we conclude that population level estimates of BPA exposure and resulting health outcome are unlikely to differ with use of the indirect vs direct method of laboratory analysis.

Measurement of free and conjugated BPA in the general population (Andra et al., 2016; Koch et al., 2017; Liao and Kannan, 2012) and in pregnancy are scarce. Several studies

have measured free BPA in pregnant women (Casas et al., 2013; Gerona et al., 2016; Guidry et al., 2015; Kubwabo et al., 2014) but only two previous studies have compared results from the same urine sample analyzed by the direct and indirect methods (Gerona et al., 2020; Provencher et al., 2014). Our results are consistent with the 12.6% higher on average total BPA measured with the LC-MS/MS versus the GC–MS/MS methods observed in a study of 49 samples (Provencher et al., 2014) using the same methodology and laboratory as those in our MIREC study. Provencher and colleagues (2014) reported median specific gravity adjusted BPA concentrations of 0.904 μg BPA equivalent /L and reported that free BPA was 1.7% of the total BPA. These authors identified possible interference in the enzymatic deconjugation due to the dietary isoflavone resveratrol glucuronide in the direct (LC-MS/MS) method measurement of BPA conjugates (Provencher et al., 2014). The BPA glucuronide and the trans-resveratrol glucuronide have about the same molecular weight (404.41 and 404.37 respectively) and the same molecular structure. Therefore, an appropriate pH (11.0) in the mobile phase and sufficient chromatography gradient are necessary to adequately separate this interference from BPAG. This step was done in the analysis presented in this manuscript (Arbuckle et al., 2015). In a review of analytic methods for BPA measurement, Andra et al (2016) similarly noted that the direct measurement of BPA conjugates is subject to interference from compounds with similar molecular weights as the conjugates, such as resveratrol and its metabolites.

Our findings contrast with those from a study of 29 pregnant women where their reported direct method geometric mean Sum BPA was 51.99 ng/mL and the indirect deconjugation-based method geometric mean Total BPA was 2.77 ng/mL (Gerona et al., 2020). These authors noted that the greatest observed disparity between the two methods was at the highest concentrations. In contrast, we observed the magnitude of the percent differences in the Bland-Altman plots was highest at the lowest concentrations likely due to imprecision near the LOD. In an earlier study of 112 pregnant women using the same direct method for BPA measurement, Gerona and colleagues reported median Sum BPA concentrations of 4.61 μg/L with free BPA representing 14% of the total derived concentration (Gerona et al., 2016). In MIREC, free BPA median concentrations were below the limit of detection, the median Sum BPA (direct LC-MS/MS method) was 0.89 μg/L and free BPA was 0.83 % of the total derived concentration (Arbuckle et al., 2015). Median total BPA concentrations have previously been reported to be higher in the US (Braun et al., 2011; CDC, 2019; Harley et al., 2013) than Canada (Arbuckle et al., 2015); however, laboratory methods (all indirect) were not identified as the reason for these differences (Lakind et al., 2012).

In response to Gerona and colleagues' (2020) conclusion that the indirect method underestimates total BPA concentrations, Calafat and Koch (2020) noted that proficiency testing performed by external quality assessment programs such as G-EQUAS (Erlangen, Germany) and the European Union HBM4EU of international laboratories using urine control samples spiked with known concentrations of free BPA and BPAG have consistently reported that results fall within the tolerance range (Calafat and Koch, 2020). Results (7% (5 out of 70 from 30 international participants)) that were outside the tolerance range were above rather than below these limits. As previously noted, our INSPQ laboratory participates in OSEQAS, another quality assessment scheme. Taken together, these rigorous quality assurance results do not support the premise expounded by Gerona and colleagues

(Gerona et al., 2020) that the indirect GC–MS/MS method underestimates total urinary BPA concentrations.

Contamination may be present in all stages of the laboratory analysis including instrumentation, laboratory environment, and interference with other compounds (Ye et al., 2013). Moreover, precise and accurate exposure assessment is particularly challenging for measurement of short-lived, ubiquitous chemicals, during times of rapid hormonally-dependent tissue development such as pregnancy (Moya et al., 2014). Both methodologies for measuring total BPA have distinct complexities and challenges. The indirect method of BPA measurement is dependent upon complete enzymatic deconjugation for accurate measurement of Total BPA. Free BPA is challenging to measure because it is rapidly and mostly glucuronidated by the liver after oral absorption resulting in a minimal amount of free circulating BPA and low detection rates (Koch et al., 2012; Kubwabo et al., 2014). Furthermore, detection of free BPA may result from contamination or from deconjugation of BPAG (Dekant and Völkel, 2008). Measurement of BPAG is less subject to external contamination than free BPA because it is endogenously produced in the liver. However, direct measurement of BPA conjugates must account for potential interference from unknown conjugates with similar molecular weights (Andra et al., 2016). Accurate reporting of BPAG concentrations may be additionally influenced by hydrolysis of BPAG that occurs in urine at neutral pH and room temperature (Waechter et al, 2007). Last, the use of an inappropriate internal standard that has a different chemical structure than the measured analyte could also contribute to an under- or overestimation of the BPA glucuronide (Provencher et al., 2014). In addition to these complexities of measuring total BPA, urine from pregnant women may contain endogenous compounds (ie digoxin-like immunoreactive factors) that could interfere with the β-glucuronidase activity and subsequently alter BPAG concentrations (Homma et al, 1991). The potential unique nature of urine collected during pregnancy and its impact on chemical concentrations warrants further investigation; however, it does not alter our conclusion regarding the comparability of the direct and indirect methods.

While we cannot exclude the potential for contamination due to free BPA in our results, meticulous attention to sample collection procedures and analysis minimized the potential for misclassification bias. In addition to the quality control measures described in the methods, several aspects of our study design helped ensure and validate data quality. First, we collected urine samples during the first trimester. The availability of first trimester urinary measurements lessens potential measurement error as samples were collected before pregnancy-related physiological changes such as increased glomerular filtration rate could have a notable influence on urinary BPA concentrations. In contrast, the 29 samples in the previously described comparison paper were collected in the second trimester (Gerona et al., 2020). Second, the methods used to ensure separation of the conjugate and resveratrol glucuronide minimizes the opportunity for this compound to influence our results.

Third, we confirmed the accuracy of results from the indirect GC–MS/MS method using a quality control step on samples that exceeded the 95% limits of agreement in the Bland-Altman plots. Twenty-five of the 32 samples that exceeded that limits of agreement were analyzed by a new LC-MS/MS BPA analogues method developed for a different MIREC

project. This new method uses a deconjugation step with the same hydrolysis enzyme as the GC–MS/MS method, but incubates for 4 instead of 3 h. Among these 25 samples, 7 had concentrations lower than the indirect GC–MS/MS method with deviations between –33 to −98% but validated results from the direct LC-MS/MS method with deviations between 2.6 and 17.2%. These findings suggest a possible occurrence of BPA contamination in the GC–MS/MS indirect analytical process for these samples. Sixteen results obtained with the new LC-MS/MS indirect method were comparable to the GC–MS/MS results (0.4 to 15.3 % of deviation), but were significantly lower than those from the direct LC-MS/MS method indicating, for both indirect methods, potential incomplete hydrolysis of BPAG in these particular samples. We were not able to draw conclusions for two samples analyzed with the GC–MS/MS method due to homogeneity or deconjugation issues. We provide details of the new indirect LC-MS/MS method and comparison results in Supplemental Material.

The uncertainty for both methods (direct LC-MS/MS vs indirect GC–MS/MS), as calculated by twice the inter-day precision, was comparable suggesting that the potential for differences between the methods due to variability in laboratory technique is low. Specifically, uncertainty was 13.2% for Total BPA (GC–MS/MS indirect method) at 1.4 μg BPA equivalents/L. This is comparable to the uncertainty at equivalent concentration of BPAG (12.8% at 2.0 μg BPA equivalents/L) which mainly represents the sum of BPA concentrations derived from the LC-MS/MS direct method. Uncertainties for the other less detected BPA conjugates were as follows: 20.7% for BPAS at 0.21 μg BPA equivalents/L, 17.7% for BPADS at 1.2 μg BPA equivalents/L and 11.8% for free BPA at 0.22 μg/L. The mean deviation (8.6%) between methods is within the uncertainty of both methods suggesting that other factors are responsible for these observed differences.

We propose four hypotheses to explain the rather small but observable discrepancy in results between the direct and indirect methods in our urine samples. First, the indirect GC–MS/MS method may have underestimated Total BPA concentrations in samples with elevated urinary specific gravities due to incomplete enzymatic deconjugation. We hypothesize that urines with higher specific gravities could generate a less adequate environment for optimum enzymatic deconjugation of BPAG. Urines with SG equal to or in excess of 1.02 may contain or produce inhibitors (e.g., ascorbic acid) that can inhibit the β-Glucuronidase enzyme's ability to hydrolyse BPAG (Taylor et al., 2017; Young et al., 1990). Our observation of more pronounced bias in samples with elevated SG compared to urines of SG < 1.02 supports this hypothesis. It is possible to assess enzymatic efficiency by adding a labeled BPA-glucuronide but this approach was not available at the time the MIREC samples were analyzed. Twenty-eight percent of participants had urine SG in excess of 1.02. Four percent of samples had a SG in excess of 1.02 and were below the LOQ of the GC–MS/MS method. Second, imprecision in both methods at concentrations near the LOD is another contributor to different results. In cases where the result from one method was below the LOD and the other result was above the LOD, percent differences exceeded 70%. Thirdly, another possible source of deviation may stem from the water content of the BPAG standard, which was not available at the time of our analysis. Currently, BPAG standard's water content from the same supplier is 10.5% and we hypothesize that the standard source used for quantification in our study had the same amount of water at the moment of the analyses. It is, therefore possible, that BPAG concentrations were overestimated because

BPAG represents 94.6% of the total BPA and the water content was not taken into account during the BPAG calibration. Considering this hypothetical bias, if we applied a −10.5% correction to LC-MS/MS BPAG results, both methods would be even more comparable than observed in our analyses. Lastly, the direct LC-MS/MS laboratory analysis was performed months after the indirect GC–MS/MS analysis and after an additional freeze–thaw cycle. The effect of multiple freeze–thaw cycles is unlikely to impact the stability of BPA as Total BPA concentrations measured in unspiked positive urine samples (n = 14) subjected to four freeze–thaw cycles were stable with deviations less than 5.0% (unpublished data).

Our ability to effectively compare the direct and indirect methods of BPA measurement was strengthened by our large sample size and use of biobanked samples from the same study participants. Furthermore, 1879 out of the 1891 women had both free and conjugated BPA measurements and Total BPA measurements; thus, the potential for selection bias in our results is minimal.

5. Conclusion

In this study of Canadian women, first trimester urinary Sum BPA concentrations measured by the direct LC-MS/MS method were slightly higher (mean percent difference 4.0% than Total BPA concentrations measured by the indirect GC–MS/MS method in samples with urine specific gravity less than 1.02, and moderately higher (20% deviation) in samples with urine specific gravity equal to or above this threshold. The level of observed differences is consistent with an acceptable level of deviation due to use of different laboratory equipment, analysts, timing, and measurement error. Our findings will help resolve the concern raised by previous authors that risk assessments based on the GC–MS/MS indirect method are dramatically underestimated (Gerona et al., 2020, 2016). Our findings also demonstrate the inherent complexities, pros and cons of each methodological approach. The MIREC study provided the opportunity to rigorously evaluate agreement between two laboratory methods for measuring BPA in the largest identified sample of pregnant women with available data on free and conjugated BPA in the same urine samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BPA	bisphenol A
BPADS	bisphenol disulfate
BPAG	bisphenol glucuronide
BPAS	bisphenol monosultate
CTQ	Centre de Toxicologie du Québec
GC–MS/MS	gas chromatography mass spectrometry
LC-MS/MS	liquid chromatography mass spectrometry
INSPQ	Institut national de santé publique du Québec
LOD	limit of detection
LOQ	limit of quantification
MIREC	Maternal-Infant Research on Environmental Chemicals study
SG	specific gravity
MRM	multiple reaction monitoring

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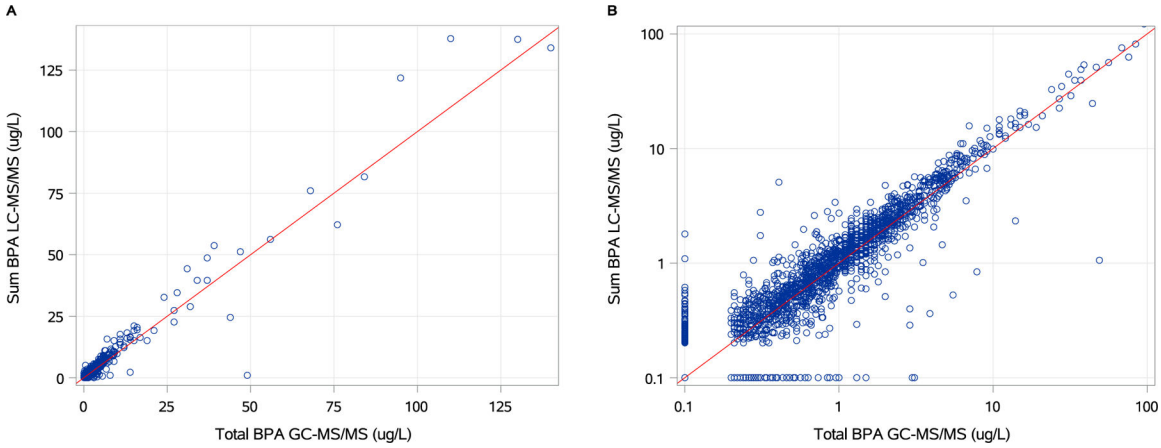

Fig. 1.

Scatterplot of Total BPA (indirect GC–MS/MS) (μg/L) and Sum BPA (direct LC-MS/MS) (μg BPA equivalents/L) with identity diagonal in red (panel A), and with a log-10 scale for both axes (panel B). LOD = 0.2 μg/L, LOD/2 imputation.

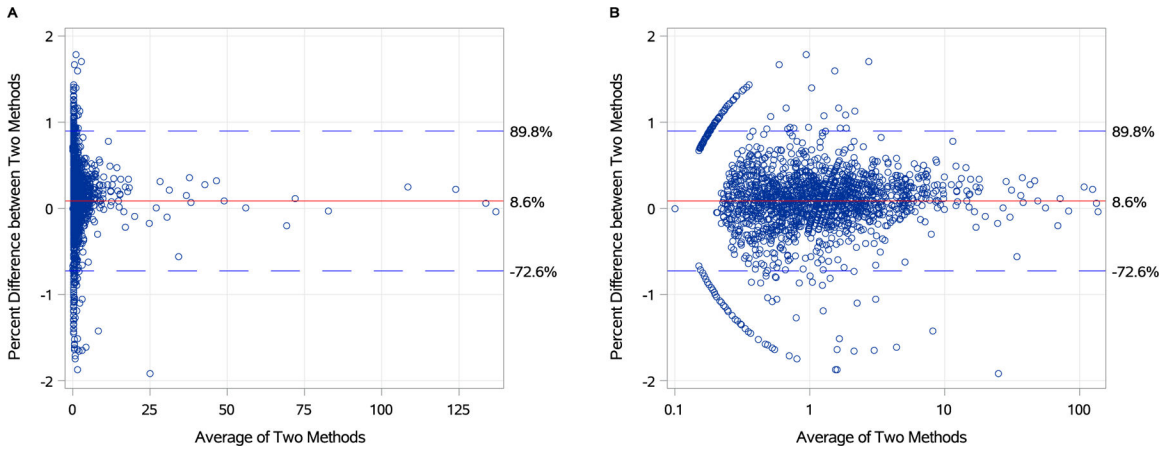

Fig. 2.

Bland-Altman analysis of percentage differences (panel A), and *x*-axis in log-10 scale (panel B). LOD = 0.2 μg/L, LOD/2 imputation.

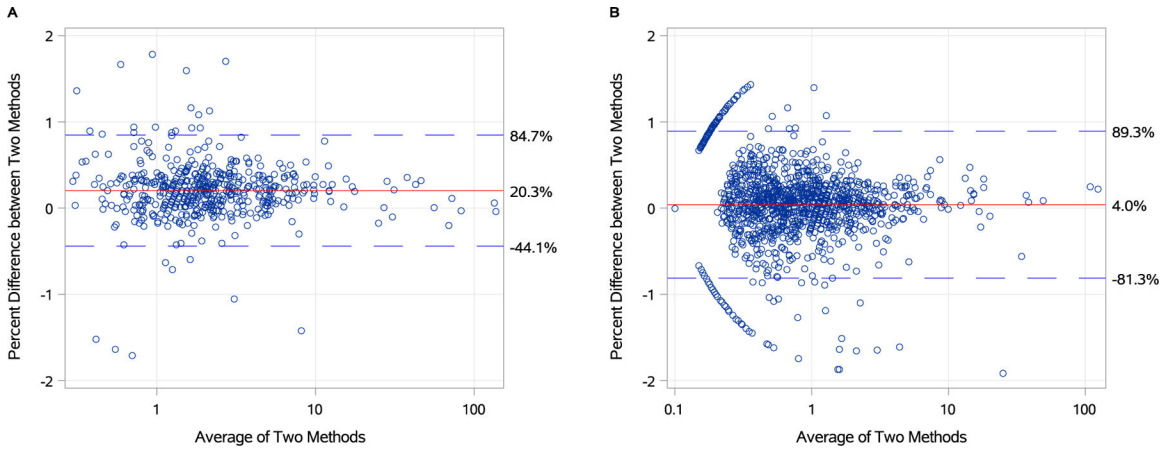

Fig. 3.

Bland-Altman analysis of percentage differences stratified according to urinary specific gravity ≥ 1.02 (panel A) and <1.02 (panel B) with x axis in log-10 scale.

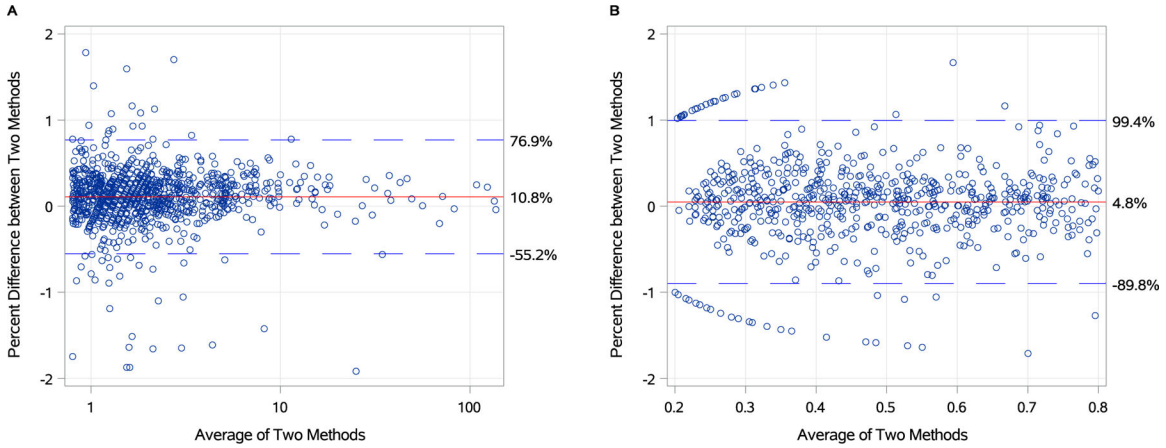

Fig. 4.

Bland-Altman analysis of percentage differences stratified according to values equal to or above LOQ (≥0.8 μg/mL) with × axis in log-10 scale (panel A) and values between the LOD and LOQ (panel B) (0.2–<0.8 μg/mL).

Table 1

Descriptive statistics of Total (indirect GC–MS/MS) (μg/L) and Sum (direct LC-MS/MS) first trimester urinary BPA (μg BPA equivalents/L) among MIREC participants (n = 1879).

	LOD	%<LOD	LOQ	Min	25th Percentile	50th Percentile	75th Percentile	Max[1]
GC–MS/MS -LOD/2 imputation								
Total BPA	0.2	12.1	0.8	0.1(LOD/2)	0.37	0.81	1.7	140
LC-MS/MS-machine readings								
BPADS	0.47	100.0	1.6	0.01 (ND)	0.01(ND)	0.01 (ND)	0.01(ND)	0.36 (ND)
BPAG	0.11	5.3	0.38	0.00 (ND)	0.36	0.83	1.83	136.15
BPAS	0.03	76.6	0.099	0.00 (ND)	0.00 (ND)	0.01 (ND)	0.03 (ND)	1.79
Free BPA	0.012	56.8	0.039	0.00 (ND)	0.00 (ND)	0.01 (ND)	0.03	2.82
Sum BPA	0.11	3.7		0.02 (ND)	0.39	0.89	1.91	137.82
LC-MS/MS-LOD/2 imputation, LOD = 0.2								
Sum BPA	0.2	10.8		< LOD	0.39	0.89	1.91	137.82

BPA: bisphenol A, BPADS: BPA disulfate, BPAG: BPA glucuronide, BPAS: BPA monosulfate, LOD limit of detection, LOQ limit of quantification, ND: below the level of detection.

[1]Statistics where all values are below the limit of detection should be interpreted with caution.

Table 2

Bland-Altman percent differences between Sum (direct LC-MS/MS) and Total (indirect GC–MS/MS) first trimester urinary BPA concentrations (n = 1879).

Mean of Differences		95% Limits of Agreement			
Mean	**95% CI**	**Lower**	**95% CI limit**	**Upper limit**	**95% CI**
8.6%	(6.7%, 10.5%)	−72.6%	(−75.8%,−69.3%)	89.8%	(86.5%,93.0%)

BPA bisphenol A.

Table 3

Bland-Altman percent differences between Sum (direct LC-MS/MS) and Total (indirect GC–MS/MS) BPA according to urine specific gravity.

	N	GM of Total BPA (95% CI)	GM of Sum BPA (95% CI)	Mean of percent differences (95% CI)	Limits of agreement	
					Lower limit (95% CI)	Upper limit (95% CI)
SG ≥ 1.02	528	1.87 (1.72, 2.03)	2.31 (2.13, 2.50)	20.3% (17.5%, 23.1%)	−44.1% (−49.0%, −39.3%)	84.7% (79.9%, 89.6%)
SG < 1.02	1348	0.57 (0.54, 0.61)	0.59 (0.56, 0.63)	4.0% (1.7%, 6.3%)	−81.3% (−85.3%, −77.3%)	89.3% (85.2%, 93.3%)

BPA bisphenol A, GM geometric mean, SG specific gravity.