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Research Article

Determination of bisphenol-A levels in human amniotic fluid samples by liquid chromatography coupled with mass spectrometry

Bisphenol A (BPA) is one of the environmental endocrine-disrupting chemicals used widely in common consumer products. There is an increasing concern about human exposure to BPA, particularly in fetuses, due to the potential adverse effects related to the estrogenic activity of BPA. In assessing environmental exposure to BPA, it is essential to have a sensitive, accurate, and specific analytical method, particularly for low BPA levels in complex sample matrices. In this study, we developed and validated an accurate, sensitive, and robust liquid chromatography–mass spectrometry (LC-MS) method for determining the BPA concentrations in human amniotic fluid (AF). In this method, BPA and the internal standards $^{13}C_{12}$-BPA were extracted from 500 μL of human AF using solid-phase extraction. Calibration curves were linear over a concentration range of 0.3–100 ng/mL for BPA. The analytes were quantitatively determined using LC-MS operated in a negative electrospray ionization selected ion monitoring mode. This validated method has been used successfully in the clinical sample analysis of BPA in second-trimester AF specimens.

Keywords: Amniotic fluid / Bisphenol A / LC-MS / SPE / Quantitation
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1 Introduction

Bisphenol A (BPA) is a widely used, endocrine-disrupting compound found in numerous household products, many of which are food-contact products [1]. Human exposures to BPA and their potential adverse health effects have caused great concern in recent years [2]. Ninety-two percent of urine samples collected from a U.S. reference population contained detectable BPA, and to a lesser extent, BPA has also been detected in saliva, maternal blood, placental tissue, and amniotic fluid (AF) [3–6]. Owing to its weak estrogenic effect, multiple studies have reported that BPA has potential to alter the normal function of the endocrine system in animals and humans [7–9]. BPA was also shown to be able to cross the placental barrier, leaving the fetus exposed to free BPA [10, 11]. Human fetuses, neonates, and infants may be more vulnerable to BPA's effects, due to their developing organs/tissues and lower capacity to detoxify compounds like BPA. BPA is mainly metabolized through glucuronidation, which is considered as a deactivation step since BPA glucuronide is devoid of BPA's estrogenic activity. However, glucuronidation capacity is not well developed in early life [12], and therefore, the limited hepatic capacity to deactivate BPA would increase the potential of fetus exposure to free BPA. Studies have shown that fetal exposure to BPA may lead to behavioral changes in offspring [13].

In assessing human environmental exposure to BPA, it is essential to have a sensitive and specific analytical method, particularly for low BPA levels in complex sample matrices, such as AF, breast milk, or meconium. Earlier analytical methods developed for the detection of BPA using enzyme-linked immunosorbent assays (ELISAs) [4, 14], HPLC with fluorescence (both with and without fluorophore derivatization) [15] and with electrochemical detections [16] suffered from the lack of sufficient specificity, especially at low levels of BPA. ELISA can be affected by the interferences of matrix effects (ME) or nonspecific binding of anti-BPA antibody [17]. The inclusion of internal standard (IS) in these methods is also difficult because of its possible interference with the detection of BPA. Gas chromatography (GC) coupled with mass spectrometry (MS) [6]

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Abbreviations: AF, amniotic fluid; **BPA**, bisphenol A; **LLOQ**, lower limit of quantitation; **ME**, matrix effect; **MRM**, multiple reaction monitoring; ***m/z***, mass-to-charge ratio; **QC**, quality control

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allows specific distinction of BPA from endogenous corticosteroids; however, the derivatization steps can be time-consuming, and additional cleanup steps are often required in order to improve method sensitivity. LC-MS does not require derivatization, so the workload for sample preparations is reduced. It selectively monitors one of the representative ions of BPA and offers the advantage of high selectivity and sensitivity. To our knowledge, the analytical method using LC-MS combined with SPE for the determination of BPA in human amniotic samples has not been previously established. The previous analytical methods were limited to using ELISA [4, 14] or HPLC-electrochemical detection [18].

The objective of the work described here was to develop and validate a sensitive, accurate, and robust LC-MS method used for the determination of BPA in human AF. The current method we developed and validated used less sample volume and exhibited higher sensitivity than the previous methods described for the quantitation of BPA in human amniotic matrix. After successful development and validation of this method, it was utilized in analyzing a set of human AF samples collected during the second-trimester period for evaluating potential fetal BPA exposure. The assay has demonstrated accuracy, reproducibility, and rigor in this analysis.

2 Materials and methods

2.1 Chemicals and reagents

HPLC-grade reagents, including acetonitrile (ACN), methanol, and water, were purchased from VWR (Bridgeport, NJ, USA). BPA, 4-methylumbelliferone, 4-methylumbelliferyl glucuronide, and β-glucuronidase/sulfatase (*Helix pomatia*, H1), concentrated ammonium hydroxide, ammonium acetate, and formic acid (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The isotope-labeled internal standards $^{13}C_{12}$-BPA (99%) and $^{13}C_4$-4-methylumbelliferone solutions were obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). Stock solutions (100 ng/mL) were prepared in methanol and used for further dilutions. The solutions were stored at −20°C. Frozen human AF (pooled from 16 individuals) and human urine (pooled from 20 individuals) were shipped from Bioreclamation (Hicksville, NY, USA) under dry ice and stored at −20°C until use. To avoid contaminations from environmental sources of free BPA, we used glassware in the lab as much as possible.

2.2 Instrumentation

The LC-MS system consisted of a Surveyor® liquid chromatographic system, containing a Surveyor MS pump and a Surveyor auto-sampler, coupled with an LCQ Deca XP (Thermo Scientific, San Jose, CA, USA) equipped with an ion trap mass analyzer and an electrospray ionization (ESI) source. The Thermo Scientific Xcalibor® software version 1.4 was used for the data acquisition and processing.

The chromatographic separation was performed on a Synergy Polar RP column (100 mm × 2.0 mm, 4 μm particle size, Phenomenex, Torrance, CA, USA) maintained at the ambient temperature with a Betasil C_{18} pre-column (10 mm × 2.1 mm, 5 μm particle size, Thermo Scientific, Madison, WI, USA). The mobile phases A and B consisted of water and ACN with 10% methanol v/v, respectively. The analysis for BPA was achieved using the following gradient program at a flow rate of 220 μL/min for 12 min: 20% B for 2 min; increased to 95% B from 2 to 5 min, and then maintained at 95% B from 5 to 8 min, decreased to 20% B from 8 to 9 min and maintained at this proportion from 9 to 12 min. The separation for 4-methylumbelliferone and 4-methylumbelliferyl glucuronide was achieved at a flow rate of 220 μL/min using the following gradient program for 12 min: 4% B from 0 to 2 min; increased to 97% B from 2 to 4 min, and then maintained at 97% B from 4 to 8 min, decreased to 4% B from 8 to 9 min and maintained at this proportion from 9 to 12 min.

The eluent from the HPLC column was directed into the electrospray probe operated in the negative-ion mode. The optimum operating conditions of the ESI were as follows: sheath gas and auxiliary gas (N_2, 99.995%, Airgas, Radnor, PA, USA) flow rates 40 a.u. (arbitrary units) and 15 a.u., respectively; capillary heater temperature 320°C; electrospray needle voltage −4.0 kV; tube lens offset voltage −60.0 V. Analytes were quantified using SIM mode with *m/z* 227 for BPA and *m/z* 239 for $^{13}C_{12}$-BPA (IS). $[M-H]^- = 175$ for 4-methylumbelliferone, 179 for $^{13}C_4$-4-methylumbelliferone (IS) and 351 for 4-methylumbelliferyl glucuronide were also selected as detecting ions.

The peak areas of analytes and their ISs were determined using Xcalibor® software version 1.4. For each analytical batch, a calibration curve of BPA with slope, intercept, and correlation coefficient (R^2) was derived from weighted ($1/y^2$) linear least-squares regression of the peak area ratio (analyte: IS) versus the concentration of the standards. The calibration curve was accepted with $R^2 > 0.98$. The regression equation from the calibration curve was used to back-calculate the measured concentration of each standard and quality control (QC). The results were compared with the theoretical concentrations to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard and QC measured. The peak area ratios of 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone and 4-methylumbelliferyl glucuronide/$^{13}C_4$-4-methylumbelliferone were used to check the extent of the deconjugation reaction.

2.3 Preparation of standard and QC samples

The initial stock standard solutions of BPA and 4-methylumbelliferone were prepared by dissolving the accurately weighted standard compounds in methanol and

quantitatively transferring this solution into a 10-mL volumetric flask. This initial stock standard solution was stored in a glass vial at −20°C until use. The intermediate standard solutions BPA and 4-methylumbelliferone were prepared by serial dilutions of the initial stock standard solution with methanol. The intermediate IS solution of $^{13}C_{12}$-BPA and $^{13}C_4$-4-methylumbelliferone were prepared by aliquoting commercial standard solution (100 μg/mL in ACN) to each 10 mL glass volumetric flask, and then diluting to the volume with methanol to give a final concentration of 1 μg/mL for $^{13}C_{12}$-BPA and 250 ng/mL for $^{13}C_4$-4-methylumbelliferone. All standard solutions were prepared in methanol-rinsed and dried glassware.

The calibration curves of BPA at eight levels ranging from 0.25 to 100 ng/mL were prepared by adding aliquots of intermediate standard solutions to diluted AF (pooled human AF/water at 1:1, v/v). The QC samples were prepared the same way in pooled human AF. The standards and QCs were stored frozen at −20°C. The enzyme solution was prepared fresh daily for each run by dissolving 4.3 mg of β-glucuronidase/sulfatase (*H. pomatia*, 1 926 000 units/g of solid) in 1 mL of 1 M ammonium acetate buffer solution (pH 5.0), and mixing gently to prevent denaturation of the enzyme.

2.4 Sample extraction

The 12-port PrepSep SPE vacuum manifold (Fisher Scientific, Pittsburgh, PA, USA) and SampliQ SPE cartridges packed with 200 mg of silica-based bonded C_{18} material (3cc) (Agilent Technology, Santa Clara, CA, USA) were used for the extraction. Amniotic samples were thawed to room temperature and vortexed to ensure homogeneity. Each of the 500 μL AF was then transferred into a glass test tube and mixed with 10 μL intermediate IS solution (1000 ng/mL of $^{13}C_{12}$-BPA), 150 μL ammonium acetate buffer solution (1 M, pH 5.0) and 30 μL of enzyme solution followed by a brief vortex to ensure mixing. The samples were then incubated at 37°C overnight in a shaking water bath. Calibration standards, QCs, including 4 at each concentration of 0.9, 10, and 50 ng/mL, and blanks were prepared and incubated the same way as amniotic samples by adding 10 μL intermediate IS solution, 150 μL ammonium acetate buffer solution, and 30 μL of enzyme solution, except that calibration standards were not incubated overnight. After incubation, 10 μL of formic acid was added to each deconjugated amniotic samples, vortexed well, and diluted with 400 μL of water. The calibration standards and QCs were processed in the same way as deconjugated amniotic samples by adding 10 μL of formic acid and diluting with 400 μL of water.

The SPE cartridges were conditioned by successive washes of 2 mL of methanol and 2 mL of water, followed by the loading of incubated amniotic samples, calibration standards, or QCs. The cartridges were washed with 1 mL of water and 500 μL of 25% ACN in water, and then eluted with 500 μL of ACN three successive times. The eluent was collected and concentrated to dryness using a TruboVap LV Evaporator (Zymark, Framingham, MA, USA) at 40°C under a gentle nitrogen stream. The dry residue was reconstituted by 110 μL ACN/water (1:1, v/v) and 20 μL was injected into the LC-MS system for the BPA analysis. The concentrations of the BPA total (free and conjugated forms) were obtained accordingly.

The concentrations of the unconjugated BPA (free BPA) were obtained by the same sample preparation procedures without deconjugation by β-glucuronidase. The concentrations of conjugated-BPA were then calculated by subtracting the concentrations of free BPA from the total-BPA. The concentration of 4-methylumbelliferone formed from the enzymatic hydrolysis of 4-methylumbelliferone glucuronide was run in parallel with the AF samples and in the purpose of monitoring the completion of the deglucuronidation reaction by β-glucuronidase. Reagent blanks, standards, and QCs were analyzed with the AF samples to ensure accuracy of the data.

2.5 Determination of BPA concentrations in AF

A total of 20 anonymous AF samples were analyzed in this study. These samples were the residual material from second-trimester samples collected originally for genetic amniocentesis, and therefore were considered as discard samples. These AF samples were collected with approval from the Brigham and Women's Institutional Review Board (IRB protocol #2010P000307). We used these materials after removing all identifiable labels with demographic information. Those samples were cell-free (supernatant) and stored at −20°C prior to the BPA analysis.

3 Results and discussion

3.1 Optimization of SPE protocol

Several LC-MS or LC-MS/MS methods have been described for the quantitative analysis of BPA in biological matrix [19–22]. The sample preparation procedures prior to LC-MS analysis in these methods include protein precipitation [19], liquid–liquid extraction [20] and mostly solid-phase extraction (SPE) [21, 22]. SPE shows advantage over the other two extraction methods not only for removing interfering components from the matrix, but also for achieving analyte pre-concentrating purpose. Since online SPE is limited by its high cost, in this study, we developed an offline SPE method. Two types of SPE cartridges Agilent Technology SampleQ C_{18} (200 mg) and Water's Oasis HLB (100 mg and 200 mg) were evaluated for the optimal adsorption and elution properties. Agilent Technology SampleQ C_{18} (200 mg) cartridge was chosen for better recoveries (~85%) than Water's Oasis HLB cartridges (<50%) at BPA concentrations of 1 and 25 ng/mL in the current

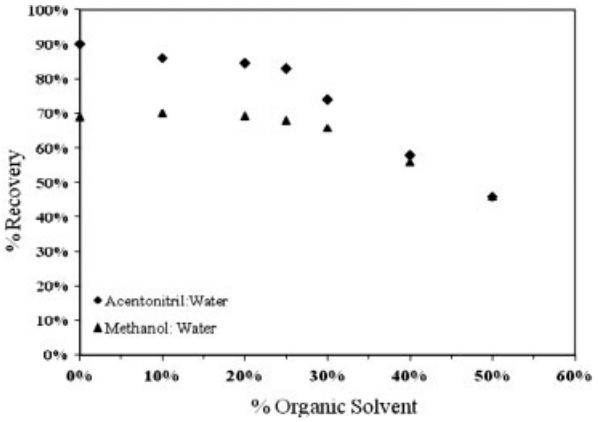

Figure 1. Influence of the composition of additional ''wash solution'' on the recovery (%) of BPA in amniotic fluid during the SPE extraction. The volume of each additional ''wash solution'' was 500 μL.

method developed. The SPE procedures were further optimized under different pHs of loading sample, wash steps, and elution solvents. Since all phenolic compounds are weak acids, acidifying samples prior to SPE will promote equilibrium to the unionized form and increases the extraction efficiency. Therefore, 10 μL of formic acid was added to the samples prior to SPE. The addition of water in the acidified samples reduced the viscosity of the sample, thus a better flow rate was achieved during SPE sample loading step.

After the sample was loaded, the SPE cartridge was subsequently washed with 1 mL of water. An additional wash step was found necessary to further clean up the samples and eliminate interferences in the analysis. Increasing proportion of methanol or ACN in water (10, 20, 25, 30, and 40%) was tested as ''wash solvent'' for this wash step (Fig. 1). ACN/water gave better recoveries than methanol/water at each proportion, and 500 μL of 25% ACN in water was ideal to wash out the interference of the sample matrix without affecting the recovery. Among the ''elution solvents'' tested, ACN elutes BPA more completely than methanol or ethyl acetate with no obvious interference. The recoveries of BPA in eluents from SPE after being dried at 25 and 40°C were also compared, and no difference was found. Thus, the sample eluents were dried at 40°C under nitrogen.

3.2 Optimization of LC-MS conditions

The negative-ion ESI mass spectra and the MS/MS production spectra of BPA and the IS were obtained by a direct infusion of the analyte solutions via a tee connection between the LC and the mass spectrometer. After the mass spectrometer operating parameters were optimized, the subsequent methods using SIM and multiple-reaction monitoring (MRM) to analyze BPA were generated and compared. SIM with *m/z* 227 for BPA and *m/z* 239 for $^{13}C_{12}$-BPA (IS) showed much stronger responses (~10-fold) than MRM with *m/z* 227/133, 227/212 for BPA and *m/z* 239/141 for $^{13}C_{12}$-BPA (IS). Therefore, the SIM mode acquisition was chosen for the quantitation of BPA in this study.

After the detection conditions were optimized, the following experiments were conducted to optimize the chromatographic conditions. We investigated several reversed-phase columns, including Water's XTerra MS C_{18} (100 mm × 2.1 mm, 3.5 μm), YMC ODS-AQ (100 mm × 2.1 mm, 3 μm), Zorbax Elipse XDB-C_8 (150 mm × 4.6 mm, 5 μm), and Phenomenex polar RP (100 mm × 2.0 mm, 4 μm). The retention time, analyte response, peak shapes, resolution, and background interferences of these columns were compared using the same mobile phase with optimized gradients. Phenomenex polar RP analytical column (100 mm × 2.0 mm, 4 μm) showed superior peak shape with adequate retention time for BPA than other columns tested. When two mobile phases, methanol/water and ACN (10% methanol, v/v)/water, were compared, the methanol/water mobile phase gave stronger signal (~1-fold) of BPA standard solution but also an interference peak with the same retention time of BPA in the blank AF samples. However, ACN (10% methanol, v/v)/water gave better resolution with no obvious interference, therefore it was chosen as the mobile phase for analyzing BPA in AF samples. No mobile phase additives, such as formic acid (0.05–0.1%), acetic acid (0.05–0.1%), ammonium acetate (5 and 10 mm), or ammonia (0.01%), were added since they caused severe signal suppression. The HPLC elution gradients and the flow rate were also optimized. The best chromatographic conditions were obtained using ACN (10% methanol, v/v) as mobile phase B starting the gradient from 20 to 95% at a flow rate of 220 μL/min. This gradient elution can separate analytes from the potential interferences with less noisy background, and gives robust and best chromatographic peaks, as shown in Fig. 2.

Stable isotopic-labeled standards (^{2}H or ^{13}C with regards to BPA) are ideal IS since they possess equal physical/chemical properties as the analyte. Because of the concern on the stability of the deuterated IS due to the possibility of exchange between deuterium and hydrogen of the solvent molecules [23, 24], we chose $^{13}C_{12}$-BPA as IS for the quantitation of BPA and added it from the beginning of the sample preparation. The addition of IS reduced the variations in sample preparation and injection, minimized the MEs on quantitation, and also improved peak identification by providing a chromatographic reference (retention time reference) for the peak selection of BPA, especially at trace-level analysis.

3.3 Calibration standards

After the selection of optimum conditions for the sample preparation and the LC-MS conditions, the method was thoroughly evaluated using calibration solutions prepared in different matrixes. Human AF is 98–99% water and the

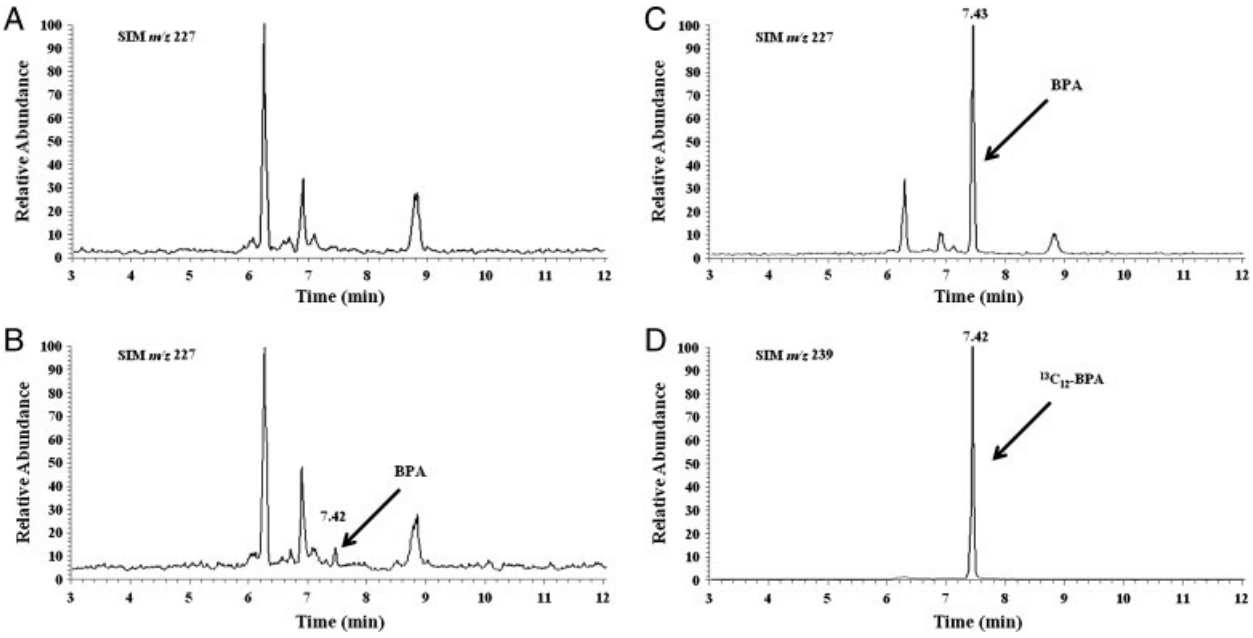

Figure 2. Representative chromatograms of: blank human amniotic fluid (A); blank human amniotic fluid spiked with 0.3 ng/mL of BPA (LLOQ) (B); 10 ng/mL of BPA (QC) (C) and 10 ng/mL of $^{13}C_{12}$-BPA (IS) (D).

Table 1. Comparison of the calibration curve slops of BPA prepared in amniotic fluid with other matrices ($n = 3$)

Matrix	Slope	% Difference[a)]	R^2
AF	0.1150		0.9970
Diluted AF solution	0.1180	2.6	0.9939
Urine	0.1441	22.4	0.9879
Diluted urine solution	0.1393	19.1	0.9899
Water	0.1270	10.0	0.9906

a) % Difference $= \frac{(\text{Mean slope} - \text{Mean slope of AF})}{(\text{Mean slope} + \text{Mean slope of AF})/2} \times 100$.

chemical composition of the fluid varies with gestational age. Up to 20 wk of gestation, AF composition is similar to fetal plasma. After 20 wk of gestation, as fetal urine production increases, urine contributes significantly to the composition of AF [25]. To evaluate potential ME on the calibration curves, the calibration standards of BPA prepared in pooled human AF were compared with water, diluted AF, urine, and diluted urine (urine/water, 1:1 v/v). The slopes of the calibrations curves in urine, diluted urine, and water showed significant differences compared with AF samples (paired *t*-test at 95% significant level), whereas the diluted AF produced calibration curves with slopes not significantly different (Table 1). This suggests that sample matrix of water, urine, and diluted urine had different influences on the sensitivity of the method as compared with AF. Therefore, calibration curves were subsequently prepared in diluted AF.

3.4 Assay validation

The experimental design and results of important criteria for method validation are presented in the following sections. The validation studies were carried out in the pooled human AF samples.

3.4.1 Linearity and LLOQ

The linearity of the calibration curve was evaluated from three consecutively prepared batches in diluted AF. The linear dynamic range of BPA was over three orders of magnitude, ranging between 0.3 and 100 ng/mL, and had correlation coefficient (R^2) exceeding 0.99 (Fig. 3). The mean back-calculated concentrations of the standards were between 90.0 and 111.7% of the theoretic values of BPA.

The limits of detection (LODs) were calculated as $3S_0$, where S_0 is the value of the standard deviation as the concentration approaches zero, and the limit of quantitation (LOQ) was calculated as 3LOD [26]. The LOD for BPA in a 500-μL AF sample was 0.1 ng/mL and the LOQ was 0.3 ng/mL. Since BPA is ubiquitous in the environment, contamination during the collection of amniotic samples in the clinics as well as the process in the laboratory analysis could result in false-positive results or over-estimated concentrations. For this reason, we have investigated the potential cross-contamination of BPA in the clinic by simulating the collection of AF samples (clinical-blank AF) in the laboratory setting using the exact clinical collecting kits (including needle, syringes, and polyethylene tubes). Laboratory blanks were also prepared to capture possible environmental contamination of BPA, such as from water or solvents, or

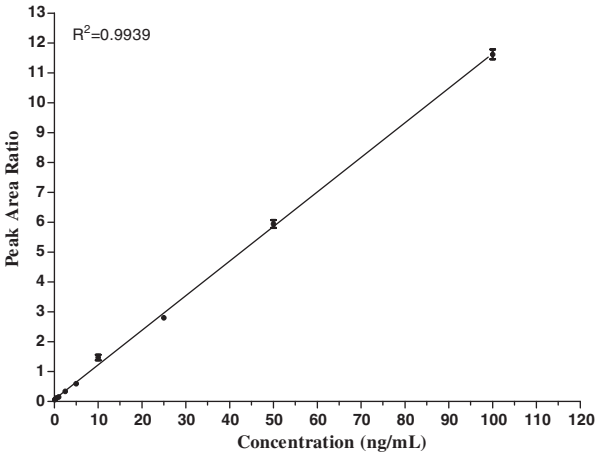

Figure 3. A calibration plot for BPA in diluted human amniotic fluid is shown. The circles represent the data points and the line represents a weighted ($1/y^2$) linear regression analysis of the data.

released from material used for sample preparation (including tubes, pipette tips, and auto-sampler vials). We found no detectable BPA in all these samples.

The sensitivity of the assay was suitable for clinical sample analysis. Twelve replicates of lower limit of quantitation (LLOQ) samples were used to evaluate the precision and accuracy at the low end of the assay range from three separate runs. The coefficient of variation (CV) was 10.9% and the accuracy, expressed as percent theoretical, was 110%.

3.4.2 Precision and accuracy

Fifteen replicates of QC samples generated at concentrations of 0.9, 10, and 80 ng/mL from runs on three consecutive days were used to evaluate precision and accuracy at each concentration level. The intra-assay CVs were between 5.0 and 9.8%, and the inter-assay CVs were between 6.4 and 8.1%. The inter-assay mean accuracies, expressed as percent of theoretical, were between 100.3 and 107.2% (Table 2). These values reflected the good accuracy and precision of the method.

3.4.3 Selectivity

Selectivity was evaluated by extracting pooled human AF samples (16 people) from three different lots and comparing the MS response at the retention times of BPA to the responses of the LLOQ. BPA and $^{13}C_{12}$-BPA regions were free from significant interferences, which suggests that the peak areas were $<20\%$ of the mean utilized LLOQs or $<20\%$ of IS response in the control zero sample. The identification of the analyte was based on its retention time and mass spectrum. In addition, the stable isotope IS served as an additional tool for the confirmation of the presence of BPA in unknown samples by providing a chromatographic reference for the peak selection.

Table 2. Descriptive statistics of human AF QC samples for assay accuracy and precision

Day	Analysis	Theoretical concentration (ng/mL) ($n = 5$)		
		LQC	MQC	HQC
		0.9	10.0	80.0
1	Within-day mean	1.0	10.6	83.1
	SD	0.11	0.7	7.9
	RSD (%)	10.9	6.9	9.5
	Accuracy (%)	109.0	106.2	100.3
2	Within-day mean	0.9	9.5	79.9
	SD	0.09	0.5	7.3
	RSD (%)	9.8	5.4	9.2
	Accuracy (%)	105.2	95.1	97.5
3	Within-day mean	1.1	10.7	82.3
	SD	0.08	0.7	4.7
	RSD (%)	7.1	6.9	5.7
	Accuracy (%)	109.6	107.2	102.9
	Overall mean ($n = 15$)	1.0	10.3	81.8
	SD	0.09	0.6	6.6
	RSD (%)	9.3	6.4	8.1
	Accuracy (%)	107.9	102.9	100.3

Table 3. ME and extraction process recovery $\pm$ standard deviation for the determination of BPA in human AF ($n = 5$)

BPA (ng/mL)	ME (%)	Recovery (%)
0.9	91.0 $\pm$ 9.7	80.3 $\pm$ 10.5
2.5	92.1 $\pm$ 3.4	84.4 $\pm$ 5.8
10	96.2 $\pm$ 2.9	89.8 $\pm$ 6.9
50	97.9 $\pm$ 8.4	86.7 $\pm$ 9.8
Mean (%) $\pm$ standard deviation	94.3 $\pm$ 6.1	85.3 $\pm$ 8.3

3.4.4 Matrix effect and extraction process recovery

ME occurs when the co-elutes from the same sample matrix affects (either attenuates or enhance) the response of the analyte during quantitation by LC-MS. To determine the ME of AF on the ESI process, blank AF samples were extracted, dried, reconstituted, and spiked with BPA (post-extraction spiked sample), and compared with the same level of concentrations of BPA in neat standard solutions injected directly into LC-MS using the following equation:

$$\text{ME}\% = (\text{Mean post extraction peak area} / \text{Mean neat solution peak area}) \times 100\%$$

The values of ME indicate either ion enhancement ($\geq 100\%$) or ion suppression ($\leq 100\%$). The results in Table 3 show the minor ion suppression (ranging from 91.0 to 97.9%) on the analyte responses. Less than 5.7% of the average intensity the analyte ions was suppressed at four

levels of BPA tested. Since the same degree of minor ion suppression was also observed in IS at each of the corresponding BPA level, the analyte/IS response ratio was unaffected across the concentration ranges, indicating that quantification of BPA using the analyte/IS response ratio was independent of the ion suppression which may directly result from the ME. These results demonstrated that the current method, including sample cleanup and HPLC separation, has adequately minimized ME on the MS ionization. This is especially important for trace-level quantification of BPA in biological matrices.

Similar to the ME, when we calculated the recovery of the extraction process using the ratio of analyte/IS, the internal standardization brought the recovery values closer to 100%. But this would not reflect the loss of sensitivity associated with poor extraction. We evaluated the recoveries of the extraction process at four levels (0.9, 2.5, 10 and 50 ng/mL) of BPA. The recovery was calculated by dividing the area counts of individual extracted AF samples by the mean area counts of neat standard solutions injected directly into LC-MS at each concentration level. Table 3 shows that the mean recovery ± standard deviation was 85.3 ± 8.3%. The mean recovery of the extraction process for IS was 81.9% (data not shown). This loss in the recovery of the extraction process is due to the loss from ME during ESI and the extraction procedure. Subtracting the 5.7% mean loss from the ME, the remaining loss from the extraction was <10%. The results demonstrated that the extraction recovery was adequate to achieve accurate, precise, and reproducible results at the LLOQ.

3.4.5 Stability

BPA stability in a biological fluid is a function of its storage conditions, which reflect situations likely to be encountered during actual sample handling and analysis, its chemical properties, the matrix, and the container system. The analyte stability in stock solution was first evaluated. The stability of standard stock solutions of BPA and $^{13}C_{12}$-BPA in methanol used in the preparation of standards and QCs was investigated both at room temperature and at −20°C as part of the validation. The stabilities were calculated by comparing mean response ratios (area of response per unit of concentration) of stability solutions to mean response ratios of freshly prepared control solutions. The stability at room temperature and −20°C of the standard stock solutions was established for at least 6 h and 60 days, respectively. The differences between the stored and fresh solutions were ≤6%, thereby indicating acceptable stability for these durations of storage for the standard stock solutions.

The stabilities of the analyte in AF during sample collection and handling, after long-term (frozen at −20°C) and short-term (room temperature) storage, and after three freeze/thaw cycles (frozen for 24 h and thawed at room temperature as one cycle) were evaluated at two concentration levels in six replicates (Table 4). The mean BPA concentration at each level was compared with each mean concentration determined in the initial testing, and found to be stable. In addition, the stability of the analyte in spiked AF under hydrolysis condition without enzyme and the stability of the processed samples in reconstitution solution

Table 4. Stability of BPA in human AF ($n = 6$)

Theoretical concentration (ng/mL)	Sample conditions									
	Bench top stability[a)]		Freeze–thaw stability[b)]		Hydrolysis stability[c)]		Processed-sample stability[d)]		Frozen matrix stability (60 days)[e)]	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
2.5	85.8	9.5	98.2	5.9	88.1	4.1	92.6	8.5	100.9	9.4
50.0	86.4	8.7	105.7	5.0	110.5	7.8	102.5	9.2	98.8	3.5

a) Room temperature for 6 h.
b) Freeze–thaw in three cycles.
c) Overnight at 37°C.
d) At room temperature (25°C) for 28 h.
e) Stored at −20°C.

Table 5. The concentrations of BPA detected in human AF samples ($n = 20$)

	BPA concentrations (ng/mL)		
	Before hydrolysis (free BPA)	After hydrolysis (total BPA)	Conjugated BPA
Range	<LOQ–0.43	<LOQ–0.75	<LOQ–0.75
Median concentration	0.38	0.47	0.26

<LOQ: below limit of quantitation.

were also evaluated. The results showed that the analyte had an acceptable stability under these test conditions (Table 4). Blank AF samples stored in polypropylene and polyethylene tubes at −20°C up to 60 days were tested and no sign of BPA contamination leaching from these tubes was found.

3.5 Assay application for clinical studies

The LC-MS method described above has been applied for the analysis of a set of AF samples collected from a group of pregnant women during the second trimester. Table 5 presents the summary of BPA levels found in these AF samples. Overall, the total BPA levels in AF were low when compared with the reported total BPA levels in urine [3]. The frequency of detection of BPA in AF samples was 80% (16 of 20 samples), slightly lower than the frequency of detection in urine samples [3]. Free BPA was detectable in 9 of 20 AF samples with a median concentration of 0.38 ng/mL, whereas the median concentrations of total and conjugated BPA were 0.47 and 0.26 ng/mL, respectively, for the 16 samples containing detectable BPA after hydrolysis. This study confirmed detectable levels of free BPA in AF samples.

4 Concluding remarks

We have developed and validated an LC-MS method for analyzing BPA in human AF with minimum interference and in relatively short chromatographic run time (12 min). The method is sensitive with an LLOQ of 0.3 ng/mL using a small sample volume of 500 μL. The success of this LC-MS method provides the efficient and timely support of clinical investigation on the association of BPA exposure and health outcomes.

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