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

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Abstract

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 BPA concentrations in human amniotic fluid. In this method, BPA and the internal standards ¹³C₁₂-BPA were extracted from 500 µL of human amniotic fluid using solid phase extraction. Calibration curves were linear over a concentration range of 0.3 to 100 ng/mL for BPA. The analytes were quantitatively determined using LC-MS operated in negative electrospray ionization selected ion monitoring mode. This validated method has been used successfully in clinical sample analysis of BPA in second-trimester amniotic fluid specimens.

Keywords

SPE; LCMS; Quantitation; Bisphenol A; Amniotic fluid

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 its 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 [3-6]. Due to its weak estrogenic effect, multiple studies

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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 exposure 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 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 behavior 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 amniotic fluid, 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 lack of sufficient specificity, especially at low levels of BPA. ELISA can be affected by the interferences of matrix effects 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] allows for 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 amniotic fluid (AF). The current method we developed and validated used less sample volume and exhibited better sensitivity than previous methods described for 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 2nd 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, methanol, and water were purchased from VWR (Bridgeport, NJ, USA). BPA, 4-methylumbelliferone, 4-methylumbelliferyl glucuronide and β -glucuronidase/sulfatas (*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 $^{13}\text{C}_{12}$ -BPA (99%) and $^{13}\text{C}_4$ -4-methylumbelliferone solutions were obtained from Cambridge Isotopes Laboratories, Inc. (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 amniotic fluid (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 a LCQ Deca XP (Thermo Scientific, San Jose, CA, USA) equipped with an ion trap mass analyzer and an electrospray ionization source. The Thermo Scientific Xcalibur® software version 1.4 was used for 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 C₁₈ pre-column (10 mm × 2.1 mm, 5 μm particle size, Thermo scientific, Madison, WI, USA). The mobile phase A and B consisted of water and acetonitrile 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, went back 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 were 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 kept at 97% B from 4 to 8 min, went back 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 electrospray ionization (ESI) were as follows: sheath gas and auxiliary gas (N₂, 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 ¹³C₁₂ BPA (IS). [*M-H*][−] = 175 for 4-methylumbelliferone, 179 for ¹³C₄-4-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 Xcalibur® 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 to 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/¹³C₄-4-methylumbelliferone and 4-methylumbelliferyl glucuronide/¹³C₄-4-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 to 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 ¹³C₁₂-BPA and ¹³C₄-4-methylumbelliferone were prepared by aliquoting commercial standard solution (100 μg/ml in acetonitrile) to

each 10 mL glass volumetric flask, and then diluting to the volume with methanol to give a final concentration of 1 $\mu\text{g/mL}$ for $^{13}\text{C}_{12}$ -BPA and 250 ng/mL for $^{13}\text{C}_4$ -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*, 1926, 000 units/g of solid) in 1 mL of 1M 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 Company, Pittsburgh, PA, USA) and SampliQ SPE cartridges packed with 200 mg of silica-based bonded C_{18} material (3 c.c.) (Agilent Technology, Santa Clara, CA, USA) were used for extraction. Amniotic samples were thawed to room temperature and vortexed to ensure homogeneity. Each of the 500 μL amniotic fluid were then transferred into a glass test tube and mixed with 10 μL intermediate IS solution (1000 ng/mL of $^{13}\text{C}_{12}$ -BPA), 150 μL ammonium acetate buffer solution (1M, 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 diluted 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% acetonitrile in water, and then eluted with 500 μL of acetonitrile three successive times. The eluent was collected and concentrated to dryness using a TurboVap LV Evaporator (Zymark, Framingham, MA) at 40°C under a gentle nitrogen stream. The dry residue was reconstituted by 110 μL acetonitrile/water (1:1, v/v) and 20 μL was injected into the LC-MS system for 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 amniotic fluid

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 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 BPA analysis.

3. Results and Discussion

3.1 Optimization of SPE protocol

Several LC-MS or LCMS/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 for not only removing interfering components from the matrix but also achieving analyte pre-concentrating purpose. Since on-line SPE is limited by its high cost, in this study, we developed an off-line SPE method. Two types of SPE cartridges Agilent Technology SampleQ C₁₈ (200 mg) and Water's Oasis HLB (100mg and 200mg) were evaluated for the optimal adsorption and elution properties. Agilent Technology SampleQ C₁₈ (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 method developed. The SPE procedures were further optimized under different pH of loading sample, wash steps, and elution solvents. Since all phenolic compounds are weak acids, acidifying samples prior to SPE extraction 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 extraction. 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 by 1mL 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 acetonitrile in water (10%, 20%, 25%, 30% and 40%) was tested as "wash solvent" for this wash step (Fig. 1). Acetonitrile/water gave better recoveries than methanol/water at each proportion, and 500 μL of 25% acetonitrile in water was ideal to wash out the interference of the sample matrix without sacrifice the recovery. Among the "elution solvents" tested, acetonitrile elutes BPA more satisfactorily 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. So 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 product-ion spectra of BPA and the IS were obtained by 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 $^{13}\text{C}_{12}$ 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 $^{13}\text{C}_{12}$ BPA (IS). Therefore, the SIM mode acquisition was chosen for 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 C₁₈ (100 mm × 2.1 mm, 3.5 μm), YMC ODS-AQ (100 mm × 2.1 mm, 3 μm), Zorbax Elipse XDB-C₈ (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 acetonitrile (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, the acetonitrile (10% methanol, v/v) : water gave good resolution with no obvious interference, so 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 acetonitrile (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 give robust and best chromatographic peaks, as shown in Fig. 2.

Stable isotopic labeled standards (²H or ¹³C with regard 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 deuteriums and hydrogen of the solvent molecules [23, 24], we chose ¹³C₁₂-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, minimize the matrix effects 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 amniotic fluid is 98-99% water and the chemical composition of the fluid varies with gestational age. Up to 20 weeks of gestation, AF composition is similar to fetal plasma. After 20 weeks of gestation, as fetal urine production increases, urine contributes significantly to the composition of AF [25]. To evaluate potential matrix effect on the calibration curves, calibration standards of BPA prepared in pooled human AF was compared with in 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 as 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 influence in 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, LLOQ, and ULOQ—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 μ L 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 released from material used for sample preparation (including tubes, pipette tips and autosampler 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 CV was between 5.0 and 9.8%, and the inter-assay CV was between 6.4 and 8.1%. The inter-assay mean accuracies, expressed as percents of theoretical, were between 100.3 and 107.2% (Table 2). These values reflected that the good accuracy and precision of the method.

3.4.3 Selectivity—Selectivity was evaluated by extracting pooled human AF samples (16 people) from 3 different lots and comparing the MS response at the retention times of BPA to the responses of the LLOQ. BPA and $^{13}\text{C}_{12}$ -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.

3.4.4 Matrix effect and extraction process recovery—Matrix effect (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 matrix effect of amniotic fluid on the ESI process, blank AF samples were extracted, dried, reconstituted and spiked with BPA (post-extraction spiked sample), and compared to 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 (> 100%) or ion suppression (< 100%). The results in Table 3 showed minor ion suppression (ranging from 91.0 to 97.9%) on the analyte responses. Less than 5.7% the average of intensity of the analyte ions were

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 were independent of the iron suppression which may directly result from the matrix effect. These results demonstrated that the current method, including sample clean up and HPLC separation, has adequately minimized matrix effect on the MS ionization. This is especially important for trace level quantification of BPA in biological matrices.

Similar to the matrix effect, 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 the mean recovery $\pm$ standard deviation was $85.3 \pm 8.3\%$. 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 likely to come from the loss from matrix effect during ESI and the extraction procedure. Subtracting the 5.7% mean loss from the matrix effect, the remaining loss from the extraction was less than 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 firstly evaluated. Stability of standard stock solutions of BPA and $^{13}\text{C}_{12}$ -BPA in methanol used in the preparation of standards and QCs was investigated at both 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 hours 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 to 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 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 found no sign of BPA contamination leaching from these tubes.

3.5 Assay application for clinical studies

The LC-MS method described above has been applied to 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 to 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. Conclusions

We have developed and validated a LC-MS method for analyzing BPA in human amniotic fluid with minimum interference and in relatively short chromatographic run time (12 min). The method is sensitive, with a LLOQ of 0.3 ng/mL using a small sample volume of 500 μ L. 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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Abbreviations

AF	amniotic fluid
BPA	bisphenol A
LOD	limit of detection
LC-MS	liquid chromatography coupled with mass spectrometer
SIM	selected ion monitoring
MRM	multiple reaction monitoring
m/z	mass-to-charge ratio

5. References

- [1]. Halden RU. Annu Rev Public Health. 2010; 31:179–194. [PubMed: 20070188]
- [2]. Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgarten FJ, Schoenfelder G. Environ Health Perspect. 2010; 118:1055–1070. [PubMed: 20338858]
- [3]. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Environ Health Perspect. 2008; 116:39–44. [PubMed: 18197297]
- [4]. Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. Hum Reprod. 2002; 17:2839–2841. [PubMed: 12407035]
- [5]. Joskow R, Barr DB, Barr JR, Calafat AM, Needham LL, Rubin C. J Am Dent Assoc. 2006; 137:353–362. [PubMed: 16570469]
- [6]. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Environ. Health Perspect. 2002; 110:A703–A707. [PubMed: 12417499]
- [7]. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Natur. 1999; 401:763–764.
- [8]. Skakkebaek NE, Rajpert-De Meyts E, Jørgensen N, Carlsen E, Petersen PM, Giwercman A, Andersen AG, Jensen TK, Andersson AM, Müller J. APMIS. 1998; 106:3–11. [PubMed: 9524557]

- [9]. Vandenberg LN, Maffini MV, Schaeberle CM, Ucci AA, Sonnenschein C, Rubin BS, Soto AM. *Reprod Toxicol*. 2008; 26:210–219. [PubMed: 18938238]
- [10]. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD. *Am J Obstet Gynecol*. 2010; 202:393.e1–7. [PubMed: 20350650]
- [11]. Zalko D, Soto AM, Dolo L, Dorio C, Rathahao E, Debrauwer L, Faure R, Cravedi JP. *Environ Health Perspect*. 2003; 111:309–319. [PubMed: 12611660]
- [12]. de Wildt SN, Kearns GL, Leeder JS, van den Anker JN. *Clin Pharmacokinet*. 1999; 36:439–452. [PubMed: 10427468]
- [13]. Braun JM, Yolton K, Dietrich KN, Hornung R, Ye X, Calafat AM, Lanphear BP. *Environ Health Perspect*. 2009; 117:1945–1952. [PubMed: 20049216]
- [14]. Yamada H, Furuta I, Kato EH, Kataoka S, Usuki Y, Kobashi G, Sata F, Kishi R, Fujimoto S. *Reprod Toxicol*. 2002; 16:735–739. [PubMed: 12401500]
- [15]. Kuroda N, Kinoshita Y, Sun Y, Wada M, Kishikawa N, Nakashima K, Makino T, Nakazawa H. *J. Pharm. Biomed. Anal*. 2003; 30:1743–1749. [PubMed: 12485715]
- [16]. Sajiki J, Takahashi K, Yonekubo J. *J. Chromatogr. B Biomed. Sci*. 1999; 736:255–261.
- [17]. Fukata H, Miyagawa H, Yamazaki N, Mori C. *Toxicol Mech Methods*. 2006; 16:427–430. [PubMed: 20021017]
- [18]. Engel SM, Levy B, Liu Z, Kaplan D, Wolff MS. *Reprod Toxicol*. 2006; 21:110–112. [PubMed: 16112541]
- [19]. Völkel W, Bittner N, Dekant W. *Drug Metab Dispos*. 2005; 33:1748–1757. [PubMed: 16103135]
- [20]. Yang M, Kim SY, Lee SM, Chang SS, Kawamoto T, Jang JY, Ahn YO. *Arch Environ Contam Toxicol*. 2003; 44:546–551. [PubMed: 12712285]
- [21]. Gallart-Ayala H, Moyano E, Galceran MT. *Rapid Commun Mass Spectrom*. 2007; 21:4039–4048. [PubMed: 18022958]
- [22]. Ye X, Kuklanyik Z, Needham LL, Calafat AM. *Anal Chem*. 2005; 77:5407–5413. [PubMed: 16097788]
- [23]. Matejczyk RJ. *J Anal Toxicol*. 1988; 12:236–238. [PubMed: 3184893]
- [24]. Verplaetse R, Tytgat J. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2010; 15; 878:1987–1996.
- [25]. Modena AB, Fieni S. *Acta Biomed*. 2004; 75(Suppl 1):11–3. [PubMed: 15301282]
- [26]. Taylor, JK. *Quality assurance of chemical measurements*. Lewis Publishers; Chelsea, M.I.: 1987.

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 μ L.

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 $^{13}\text{C}_{12}$ BPA (IS) (**D**).

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.

Table 1

Comparison of the calibration curve slopes 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

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

Table 2

Descriptive statistics of human amniotic fluid QC samples for assay accuracy and precision

Day	Analysis	<u>Theoretical Concentration (ng/mL) (n=5)</u>		
		LQC 0.9	MQC 10.0	HQC 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

Matrix effect (ME) and extraction process recovery $\pm$ standard deviation for the determination of BPA in human amniotic fluid (n=5)

BBPA (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

Table 4

Stability of BPA in human amniotic fluid (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 hours^b Freeze-thaw in three cycles^c Over night at 37°C^d At room temperature (25°C) for 28 hours^e Stored at -20 °C

Table 5

The concentrations of BPA detected in human amniotic fluids 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