Journal of Chromatography B, 953–954 (2014) 53–61

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Biomonitoring method for bisphenol A in human urine by ultra-high-performance liquid chromatography–tandem mass spectrometry

David J. Anderson[a,*], Eric M. Brozek[a], Kyley J. Cox[b], Christina A. Porucznik[b], Diana G. Wilkins[a,c]

[a] Center for Human Toxicology, University of Utah, 30 South 2000 East, Room 201, Salt Lake City, UT 84112, United States
[b] Department of Family and Preventive Medicine, Division of Public Health, University of Utah, 375 Chipeta Way, Suite A, Salt Lake City, UT 84108, United States
[c] Department of Pathology, University of Utah, 15 North Medical Drive, Salt Lake City, UT 84112, United States

ARTICLE INFO

Article history:
Received 27 September 2013
Accepted 17 January 2014
Available online 2 February 2014

Keywords:
Bisphenol A
Liquid chromatography
Tandem mass spectrometry
Human urine

ABSTRACT

An ultra-high-performance liquid chromatography–tandem mass spectrometry method for the measurement of total bisphenol A in human urine was developed and validated. The method utilized liquid/liquid extraction with 1-chlorobutane and a human urine aliquot size of 800 μL. Chromatography was performed on an Acquity UPLC® system with a Kinetex® Phenyl-Hexyl column. Mass spectrometric analysis was with negative electrospray ionization on a Quattro Premier XE™. The surrogate matrix method was used for the preparation of calibration standards in synthetic urine due to the presence of BPA in control human urine. The validated calibration range was 0.75–20 ng/mL with a limit of detection of 0.1 ng/mL. The internal standard was d_{16}-bisphenol A. Method validation utilized quality control samples at three concentrations in both synthetic urine and human urine. Bisphenol A mono-glucuronide was fortified in synthetic urine in each analytical run to monitor the enzymatic conversion of the glucuronide conjugate to BPA by β-glucuronidase. Validated method parameters included linearity, accuracy, precision, integrity of dilution, selectivity, re-injection reproducibility, recovery/matrix effect, solution stability, and matrix stability in human urine. Acceptance criteria for analytical standards and QCs were ±20% of nominal concentration. Matrix stability in human urine was validated after 24 h at ambient temperature, after three freeze/thaw cycles, and after frozen storage at −20 °C and −80 °C for up to 218 days. The method has been applied to the analysis of over 1750 human urine samples from a biomonitoring study. The median and mean urine BPA concentrations were 2.71 ng/mL and 4.75 ng/mL, respectively.

1. Introduction

There has been considerable scientific interest in bisphenol A (BPA), particularly in the research areas of environmental science [1] and human health [2–5]. With the advancement of sensitive analytical instrumentation and improved methodologies, BPA has been detected and quantified in a range of matrices, such as soil [6] and water [7–10], and human blood, urine, and saliva [11–29]. In light of the improved analytical sensitivity, the ubiquitous detection of BPA is not entirely unexpected, as this chemical is produced in large quantities [3] with applications in products as widespread as polycarbonate plastics, epoxy resins, and thermal printing paper [3,30]. In turn, the use of BPA in the manufacturing of products as common as bottles and cans, used for consumer food products, leads to human dietary exposure [4].

The initial concerns about human health effects from BPA exposure stem from the chemical's well-documented estrogenicity [3–5]. Additional concerns have been raised about exposure to BPA during fetal and early childhood development [22,31–34]. Over time, the interest of the scientific community in the human health aspects of BPA exposure has expanded with biomonitoring studies being an important part of this research [35,36]. Biomonitoring studies provide the analytical data that are a crucial component in the overall assessments of risk [37]. Concurrent with biomonitoring efforts, there has been a continued emphasis on the need for reliable analytical methods for measuring BPA concentrations in human samples.

* Corresponding author. Tel.: +1 8015815117.
E-mail address: anderson.dave@utah.edu (D.J. Anderson).

1570-0232/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2014.01.039

Human plasma, serum, and urine have been used in many biomonitoring studies to assess BPA exposure. Published analytical methods for the measurement of BPA in human samples generally fall into these categories [36–38]: immunosorbent assays (e.g., ELISA), chromatographic separation with non-mass-spectrometric detection (e.g., electrochemical, fluorescence), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (LC–MS). The GC–MS and LC–MS methods have been the methods of choice in recent years because of the limited analytical selectivity of immunosorbent and non-mass-spectrometric detection in chromatographic methods. The analysis of BPA using GC–MS requires chemical derivatization in order to impart volatility to the analyte, and this aspect has led to LC–MS becoming more common than GC–MS. Recently, ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) methods have appeared [19,27].

Regarding methods for BPA in human urine, a distinguishing feature is the chemical form of the target analyte. BPA exists in human urine primarily as the glucuronide conjugate, with the other main forms including the sulfate conjugate and unconjugated BPA [29,36], although other forms such as the disulfate conjugate and glucuronide/sulfate diconjugate have been reported [29,39]. Methods can be distinguished as measuring unconjugated BPA [18], both unconjugated and glucuronide conjugate [11,20,29], both unconjugated and total BPA [14,22], and total BPA [12,13,19,21,25–27]. Total BPA is defined as the sum of the conjugated and unconjugated forms. A typical method for total BPA in urine utilizes an enzymatic hydrolysis step with glucuronidase that also contains sulfatase activity (e.g., β-glucuronidase, Type H-1 from *Helix pomatia*) to convert the conjugated BPA to the unconjugated form prior to analysis [13].

Due to the widespread use of BPA in consumer products, as well as the presence of BPA in the environment, one of the primary analytical challenges in measuring ultra-trace concentrations (i.e., parts-per-trillion) of BPA is the presence of the analyte in control matrix samples. In the absence of an analyte-free control matrix, the analytical approach becomes similar to that of endogenous biomarkers [40]. In biomarker methods, two common approaches to overcoming the presence of the analyte in the control matrix are the surrogate analyte approach and the surrogate matrix approach. In the surrogate analyte approach, a form of the analyte that is labeled with a stable isotope (e.g., ^{13}C) is used in place of the analyte in the calibration standards and quality control samples. In the surrogate matrix approach, an alternate matrix is used. Examples of alternate matrices include native matrix from an animal, charcoal-stripped human matrix, and synthetic or buffer-based substitute matrix. In addition, background subtraction or blank subtraction can be used, alone or in combination with either surrogate approach. The method of standard addition, though labor intensive, is also applicable in some cases [40]. Regardless of the analytical approach that is used, it is critical that the method validation process include experiments to assess that acceptable sensitivity, selectivity, recovery, accuracy, and precision are obtained.

This paper presents the development and validation of a UHPLC–MS/MS method for the measurement of total BPA (unconjugated BPA, plus mono-glucuronide conjugate and mono-sulfate conjugate) in human urine following liquid/liquid extraction. The method utilizes the surrogate matrix approach with calibration standards prepared in synthetic urine. Details of the method are presented along with discussion of critical method development components, followed by results from the method validation, including results for quality control (QC) samples in both human urine and synthetic urine. A brief synopsis of the application of the method to a biomonitoring study is also presented.

2. Experimental

2.1. Materials

Bisphenol A (≥99% purity, Aldrich® brand), bisphenol A mono-β-D-glucuronide (BPA-g, ≥95% purity, Aldrich® brand), and d_{16}-bisphenol A (d_{16}-BPA, ≥99% purity, Supelco® brand) were purchased from Sigma–Aldrich® (St. Louis, MO). Bisphenol A monosulphate (BPA-s) was synthesized in-house following the method described in Shimizu et al. [41]. Water used in extractions and preparation of reagents and LC mobile phase was obtained from a Millipore Milli-Q® Gradient A10 system (Bedford, MA). Ammonium bicarbonate, ammonium acetate, and β-glucuronidase (Type H-1 from *H. pomatia*) were purchased from Sigma–Aldrich® (St. Louis, MO). LC–MS grade methanol and acetonitrile were purchased from Burdick & Jackson® (Morristown, NJ). The liquid/liquid extraction solvent, 1-chlorobutane (OmniSolv® grade), was from EMD Millipore (Billerica, MA). Acetic acid was from Spectrum® (Gardena, CA). Synthetic urine was from Immunalysis (Pomona, CA).

2.2. Instrumentation

The chromatographic system was a Waters Acquity UPLC® system (Milford, MA) including a binary solvent manager (i.e., binary pump), sample manager (i.e., autosampler), and column heater. The mass spectrometer was a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with MassLynx™ (version 4.1) software (Milford, MA). Data processing and quantitation were with TargetLynx™ software (version 4.1).

2.3. UHPLC conditions

Chromatography was performed with a Kinetex® Phenyl-Hexyl, 2.1 mm × 75 mm, 2.6 μm particle size, with a SecurityGuard™ ULTRA Phenyl, 2.1 mm × 2 mm guard cartridge (Phenomenex®, Torrance, CA), both at a temperature of 35 °C. The autosampler was set at 10 °C, and the injection volume was 25 μL in the partial loop mode. Strong and weak needle washes were acetonitrile and 5% methanol in water, respectively. Mobile phase A was 5 mM ammonium bicarbonate (pH 8.0, unadjusted), and mobile phase B was methanol using a flow rate of 300 μL/min. Gradient conditions were: 50% B for 0.5 min, increased linearly (curve = 6) to 80% B from 0.5 to 3.25 min, held at 80% B until 3.5 min, increased linearly to 100% B from 3.5 to 5.98 min. Conditions were returned to 50% B at 5.99 min, and the UHPLC run time ended at 6.0 min. The switching valve on the Quattro was used to divert the column effluent away from the ion source for the first 1.5 min and last 2.75 min of each injection. Retention times for BPA and d_{16}-BPA were approximately 2.5 min with estimated k prime values (column capacity factor) of approximately 3 (see Fig. 1).

2.4. Mass spectrometric conditions

Electrospray ionization (thermally-assisted) was used in the negative ionization mode. Ion source and MS/MS conditions were optimized by infusion of a 1 μg/mL BPA solution at 20 μL/min into a 200 μL/min mobile phase flow of 50% B. Optimal conditions were: source temperature 130 °C, desolvation temperature 400 °C, N_2 desolvation gas flow rate 800 L/h, N_2 cone gas flow rate 50 L/h, capillary voltage −2.75 kV, cone voltage −40 V, collision energy 20 eV, and Ar collision gas flow 0.16 mL/min. Nitrogen and argon were ultra-high-purity grade. The Quattro was set for unit mass resolution (FWHM approximately 0.7 amu) in both mass analyzers and operated in the Selected Reaction Monitoring (SRM) mode. Mass transitions (precursor ion → product ion) and dwell times were

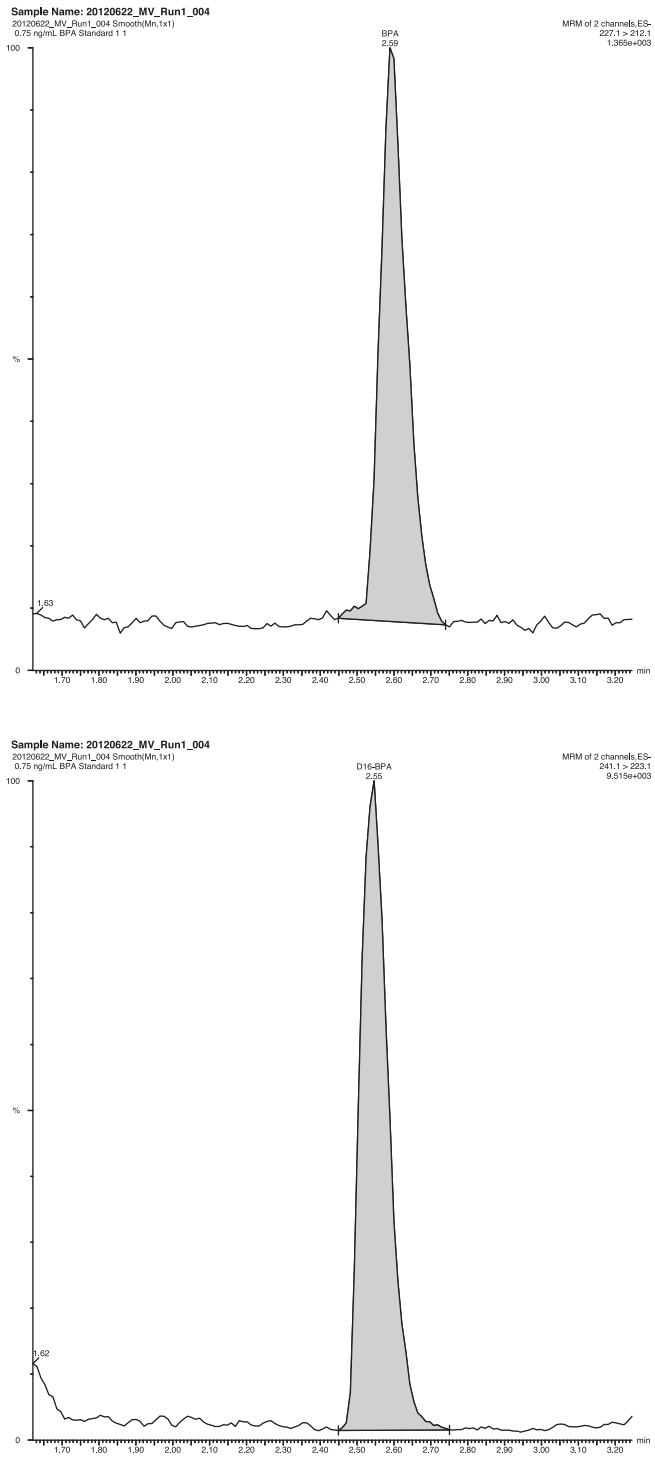

Fig. 1. SRM mass chromatogram of 0.75 ng/mL BPA standard (LLOQ) plus IS in synthetic urine.

227.1 → 212.1 and 400 ms for BPA and 241.1 → 223.1 and 200 ms for d_{16}-BPA.

2.5. Preparation of calibration standards and quality control (QC) samples

Standard stock solutions of BPA were prepared at a concentration of 1 mg/mL in methanol using a Mettler Toledo XS3DU microbalance (Columbus, OH) and Class A volumetric flasks. Working solutions were prepared at concentrations of 10, 1, 0.1, and 0.01 μg/mL in 90:10, methanol:water, as opposed to 100% methanol, to reduce the risk of concentration changes during use from solvent evaporation. All solutions were stored in polypropylene at −20 °C.

Calibration standards were prepared fresh for each analytical run in control synthetic urine. Duplicate calibration curves were fortified and extracted for each run at concentrations of 0.75, 1, 2, 4, 5, 10, 15, and 20 ng/mL. Two double blanks (no analyte or internal standard) and two zero samples (internal standard only) were prepared in control synthetic urine for each run.

The internal standard (IS) stock solution was also prepared in methanol. Dilutions to the IS working solution concentration of 100 ng/mL were made in 90:10, methanol:water.

QC stock and working solutions were prepared in the same manner as the standard solutions. However, a separate weighing of the BPA reference material was performed for use in the preparation of QCs.

During method validation, bulk QC samples were prepared in both control synthetic urine and control human urine at concentrations of 2.25, 8, and 16 ng/mL. Individual lots of the control human urine were pre-screened for BPA prior to use. Selected individual lots of control human urine were then pooled, based on the results of the pre-screening, for use in the preparation of the bulk QCs. The target concentration for BPA in the control human urine pool was between the limit of detection and the lower limit of quantitation (LLOQ). After bulk preparation and thorough mixing, 2.7 mL of each QC was aliquoted into individual polypropylene containers for frozen storage. The three QC levels were assayed at $N = 6$ during method validation and $N = 3$ during sample analysis. Three replicates of the pooled control human urine, used in the preparation of the human urine QCs, were also assayed in each run. Only QCs in control human urine were used during sample analysis.

The standard stock solution of BPA-g was prepared at a concentration of 100 μg/mL in methanol in the same manner as the BPA standard solutions. The working solution was prepared at a concentration of 0.1 μg/mL by dilutions with 90:10, methanol:water. Three replicate fortifications of 5 ng/mL BPA-g in control synthetic urine were prepared fresh and included in each analytical run to monitor the enzymatic deconjugation of BPA-g to BPA. These were the BPA-g check samples. Calculation of the percent conversion of BPA-g to BPA was based on the theoretical concentration of BPA that would result from complete conversion of a 5 ng/mL spike of BPA-g. The theoretical concentration is the ratio of molecular weights of BPA/BPA-g multiplied by the percent purity of the BPA-g reference material.

β-Glucuronidase was prepared fresh for each run by weighing the solid enzyme directly into a tared, 20 mL glass scintillation vial. The enzyme was dissolved by gentle mixing in 0.5 M ammonium acetate (pH 5.0) at a concentration of 2 mg/mL. The solid enzyme was stored at −20 °C.

2.6. Sample preparation

Human urine samples were stored at −20 °C and thawed unassisted on the first day of the extraction. Samples were thoroughly mixed by inverting and vortexing prior to aliquoting. An 800 μL aliquot of each sample, standard, QC, BPA-g check sample, double blank, and zero sample was pipetted into polypropylene test tubes. 50 μL of the 100 ng/mL IS solution was added to all tubes, except double blanks, and the tubes were vortex mixed. 200 μL of the 2 mg/mL β-glucuronidase solution was added to all tubes, followed by gentle mixing by inversion. All tubes were then incubated overnight at 37 °C in a water bath for at least 16 h. On the second day of the extraction, the tubes were removed from the water bath and allowed to cool prior to the addition of 4 mL of 1-chlorobutane to each tube. Tubes were placed on a reciprocal shaker

for 20 min, followed by centrifugation at 3200 rpm for 20 min. The lower, aqueous layer was frozen by placing racked tubes in a −80 °C freezer for approximately 30 min. The upper, organic layer was then transferred to a fresh tube for solvent evaporation in a Turbovap® (Zymark) at 40 °C. The dried extracts were reconstituted with 200 μL of 50:50, 5 mM ammonium bicarbonate:acetonitrile and transferred to conical, polypropylene autosampler vials for analysis.

2.7. Analysis and quantitation

Batch analysis was based upon standard bioanalytical practices [42] and included one set of freshly-extracted calibration standards at the beginning and the other set at the end in each analytical run. Duplicate calibration curves were analyzed in this manner, during both method validation and sample analysis, to demonstrate acceptable performance of the calibration for the duration of an analytical run. A double blank and a zero sample in synthetic urine were analyzed with each set of standards. During method validation, the six replicate QCs were grouped by concentration in the batches. For sample analysis batches, one set of low, medium, and high concentration QCs were analyzed after the first standard curve, with one set near the middle of the batch, and the third set just before the second standard curve. Three replicates of the pooled human urine used in the preparation of the human urine QCs were analyzed as zero samples after the first synthetic urine zero sample, followed by the BPA-g check samples.

Peak area ratios of BPA to the IS were used for quantitation. The calibration standards were regressed in the TargetLynx™ software using a linear model and $1/x^2$ weighting, which were chosen based on the deviations from nominal observed during method validation. Standards that did not meet the criteria were excluded from the regression.

Results were determined directly from the regression, except for the human urine QCs. Concentrations of the triplicate, pooled human urine zero samples in each batch were averaged and added to the nominal concentration for the human urine QCs for that batch. Accuracy for the human urine QCs was then calculated as the ratio of the measured concentration to the adjusted nominal concentration. Also, the average concentration of BPA from the three BPA-g check samples was used to calculate an average conversion of BPA-g to BPA for each batch.

2.8. Method development

During initial stages of method development, optimal stationary phase and mobile conditions were evaluated empirically. It was determined that the sensitivity was better with slightly basified mobile phase A, as compared to neutral or mildly acidic conditions. Also, the phenyl-hexyl stationary phase showed better retention than C_{18}. Other method parameters required more rigorous experimentation.

The type of material used for storage containers was evaluated. Solutions of BPA in 90:10, methanol:water at 10 ng/mL and 100 ng/mL were aliquoted to duplicate silanized glass test tubes and duplicate polypropylene centrifuge tubes. One solution in each container material at both concentrations was subject to three, serial transfers into the same type of tube. The solutions were held for 10 min after each transfer. After the third transfer, aliquots of the duplicate solutions were removed, fortified with IS, and analyzed. Comparison of the treated solutions (three transfers) to the untreated solutions (no transfers) showed no evidence of analyte loss during transfer for both materials at either concentration. Polypropylene was selected based upon cost and utility.

Selection of the LLOQ was based on the needs of the biomonitoring study and a review of the literature. The required sensitivity of the method was to reliably measure 1 ng/mL, so the LLOQ was chosen to be slightly lower than this value (0.75 ng/mL). Aliquot size was optimized to keep the laboratory background, which corresponded to the BPA signal in the synthetic urine double blanks and zero samples, below 20% of the response at the LLOQ. An increase in aliquot volume from 200 μL to 400 μL, and again to 800 μL, resulted in an increased response at the LLOQ without an increase in the synthetic urine negative controls, thus reducing the response in the negative controls as a percentage of the LLOQ response.

Methyl *tert*-butyl ether (MtBE) was initially chosen as the extraction solvent. MtBE showed good recovery (roughly 80%) and an acceptable chromatographic baseline response in early method development when working solely with the synthetic urine matrix. Synthetic urine was used before human urine to better evaluate method sensitivity without a contribution of the analyte from the control matrix. As part of the transition to authentic matrix, post-column infusion experiments were performed using human urine extracts to investigate matrix suppression or enhancement of ionization. These experiments showed no discernable difference in the response profile of an infused solution of analyte between an injected human urine extract or reconstitution solvent. When human urine samples were screened for use in the preparation of QC pools, an elevated chromatographic baseline was encountered for some of the individual samples. Although these problematic samples could be avoided, the implications for study sample analysis were clear, and this area required further testing. One option that was considered was the use of an alternate product ion (m/z 133) for BPA. However, a change in the SRM transition for BPA would cause a loss of sensitivity because the alternate product ion at m/z 133 was much less abundant than m/z 212, even after optimization of ionization parameters. A change of extraction solvent was a better option, and 1-chlorobutane was evaluated along with a 4:1 mixture of 1-chlorobutane:acetonitrile. The results for 100% 1-chlorobutane showed a reduced chromatographic baseline for the problematic samples, along with comparable recovery to MtBE.

Initial conditions for the enzymatic hydrolysis of BPA-g were based on published methods [12,13]. A comparison between 2 mg/mL and 4 mg/mL β-glucuronidase showed little difference for 5 ng/mL of BPA-g fortified in control human urine ($N = 3$) and incubated at 37 °C for 2 h (an estimated 102% average conversion versus 98.8%, respectively). Quantitation in this experiment was based on a single-point calibration of BPA at 5 ng/mL. Further evaluation of the hydrolysis step was done with a time series from 0.5 h to 24 h using a full calibration curve and QC samples. The time series used 5 ng/mL of BPA-g fortified in control synthetic urine ($N = 3$), 2 mg/mL of enzyme, and 37 °C incubation. Average percent conversions were 91.4% at 30 min, 90.0% after 1 h, 93.8% after 2 h, 93.2% after 4 h, and 99.6% after 24 h. These results indicated than an overnight incubation was preferable, and a follow-up experiment showed comparable results for 16 and 24 h incubations at two concentrations of BPA-g, 5 ng/mL and 15 ng/mL ranging from 99.4% to 102% average conversion ($N = 3$). Enzymatic hydrolysis testing for BPA-s showed comparable percent conversions to BPA-g fortified controls.

2.9. Method validation

Validation of the analytical method [42,43] was performed prior to the analysis of study samples. Linearity, accuracy, and precision were evaluated over the calibration range described above in three core validation runs. Chromatographic carryover was also evaluated by the placement of zero samples following both highest concentration calibration standards in each run. QCs at three concentrations in both synthetic urine and human urine were used in these runs to demonstrate reliable quantitation in either matrix against calibration standards in the surrogate matrix. Integrity of

dilution, selectivity, re-injection reproducibility, recovery/matrix effect, and matrix stability (bench-top and freeze/thaw in human urine) were also evaluated. Additional long-term matrix stability (LTMS) in human urine was conducted at nominal time points of 1, 3, and 6 months for both −20 °C and −80 °C storage temperatures. Working solution stability for BPA and BPA-g was also evaluated.

Dilution integrity was assessed with QC samples (50 ng/mL) in both synthetic and human urine for subsequent 10-fold dilution in synthetic urine. Selectivity was assessed in human urine by preparing a single QC, fortified at 2.25 ng/mL, along with a single double blank and triplicate zero samples in each of 6 different lots of control human urine. The zero sample results were used to adjust the nominal concentration of the fortified QC in each lot, as described in Section 2.7.

Re-injection reproducibility was assessed by re-injecting synthetic urine and human urine low and high QC extracts ($N = 3$) from Run 1 and quantifying them against the Run 2 calibration standards after storage in the cooled autosampler for 4 days. The combined recovery and matrix effect on ionization were determined by comparing the mean peak areas for the analyte and internal standard in extracted low, medium, and high QCs, for both synthetic urine and human urine, to mean peak areas in neat solution (i.e., reconstitution solvent). Bench-top stability was determined at low and high QC concentrations after storage at ambient temperature for 24 h. Freeze/thaw stability also used low and high QCs that were cycled three times.

BPA working solution stability (−20 °C) was assessed at nominally one year by comparison to a fresh working solution, subsequently prepared from a fresh stock solution, after appropriate dilution and LC–MS/MS analysis. Working solution stability (ambient) for BPA-g was assessed after storage at room temperature for 20 h by comparison to a control solution stored at −20 °C during the same time. The two BPA-g solutions were used to separately fortify synthetic urine at 5 ng/mL ($N = 3$) with subsequent sample preparation with and without β-glucuronidase. Any detectable BPA in the 20-h bench-top treatment without enzyme would be indicative of room temperature hydrolysis in solution.

Run acceptance criteria were based on a combination of bioanalytical [42] and forensic toxicology [43] standard practices. Calibration standards were excluded from the regression if the deviation from nominal exceeded ±20% with at least three fourths of the standards required to meet the criterion. Mean accuracy and precision criteria for the $N = 6$ QC samples were also ±20%. Criteria for BPA-g enzymatic hydrolysis check samples were ±20% from theoretical. All matrix stability results were based upon the adjusted nominal QC concentrations.

3. Results and discussion

3.1. Method validation

Results for the calibration standards were linear using $1/x^2$ weighting over the concentration range of 0.75–20 ng/mL. The selection of regression model and weighting was made after completion of the 3 core validation results and based upon inspection of deviations from nominal concentration. All individual calibration standards were within the ±20% criterion, except one of the 1 ng/mL standards in the third validation run (+25% deviation and excluded from the calibration). There was no evidence of chromatographic carryover.

Representative chromatograms of a 0.75 ng/mL BPA standard (LLOQ), a synthetic urine double blank, a synthetic urine zero sample, and a human urine zero sample are shown in Figs. 1–4. There was sufficient signal-to-noise (S/N > 10) at the LLOQ for all 0.75 ng/mL standards during method validation. The limit of

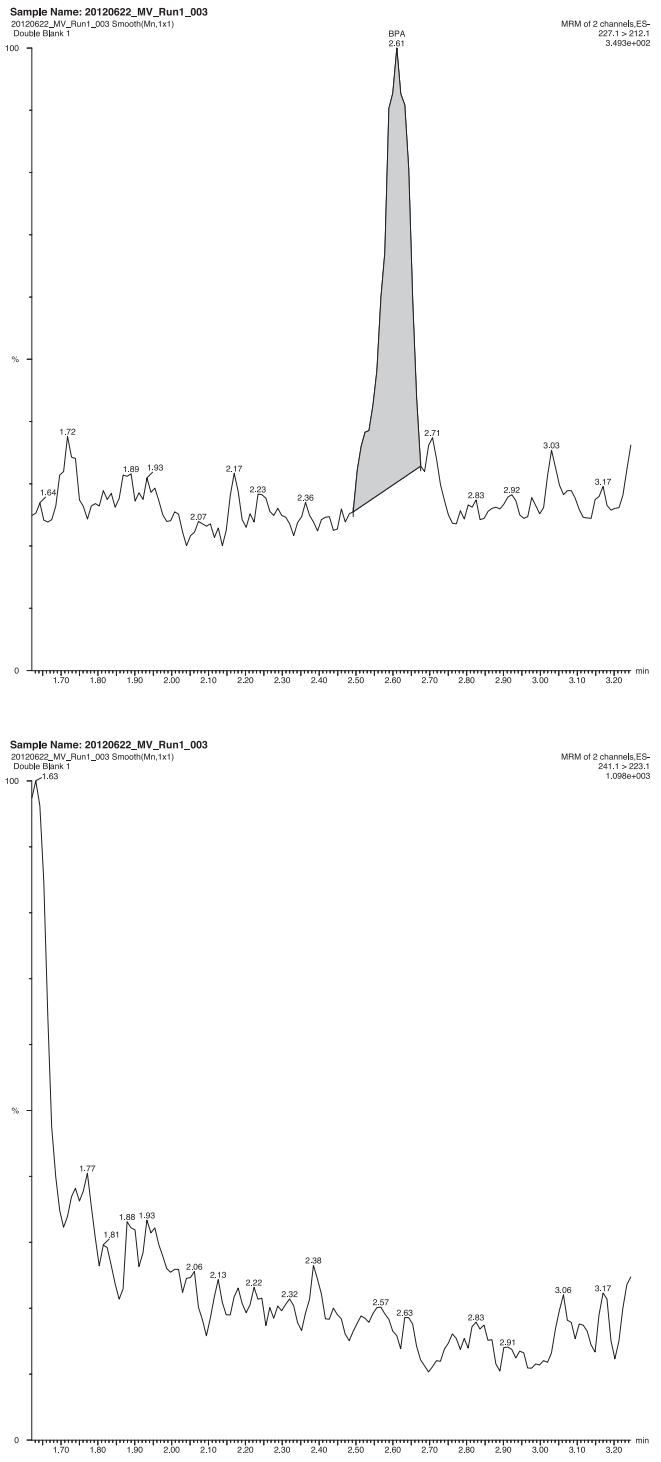

Fig. 2. SRM mass chromatogram of synthetic urine without IS (double blank).

detection (LOD) was determined by two methods, both of which required additional data from sample analysis runs because there were not enough synthetic urine zero samples with a detectable BPA signal from the first three validation runs. One method used the analyte/internal standard area ratio in the synthetic urine zero samples divided by the calibration slope (average, $N = 15$). In the second, the appropriate Student's t value was multiplied by the standard deviation of the calculated concentrations in the synthetic urine zero samples ($N = 11$). The calculated LOD by both methods was 0.1 ng/mL.

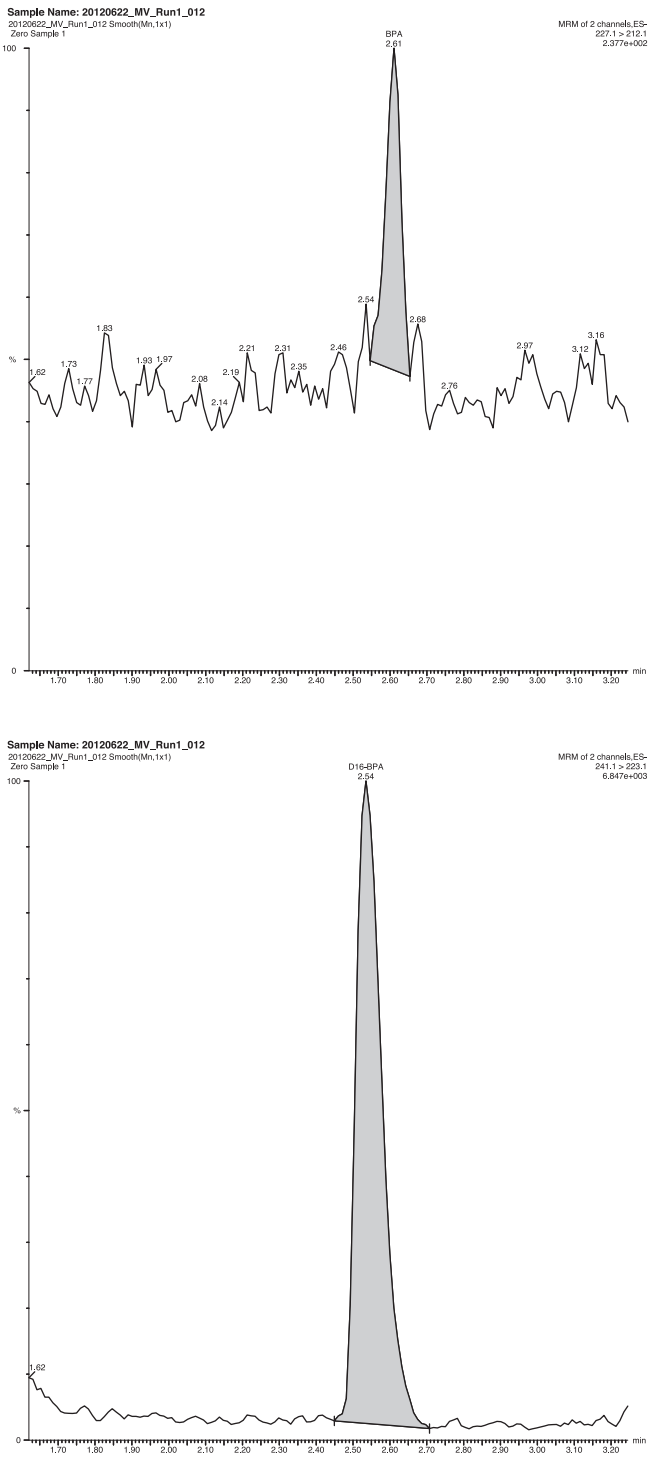

Fig. 3. SRM mass chromatogram of synthetic urine fortified with IS (zero sample).

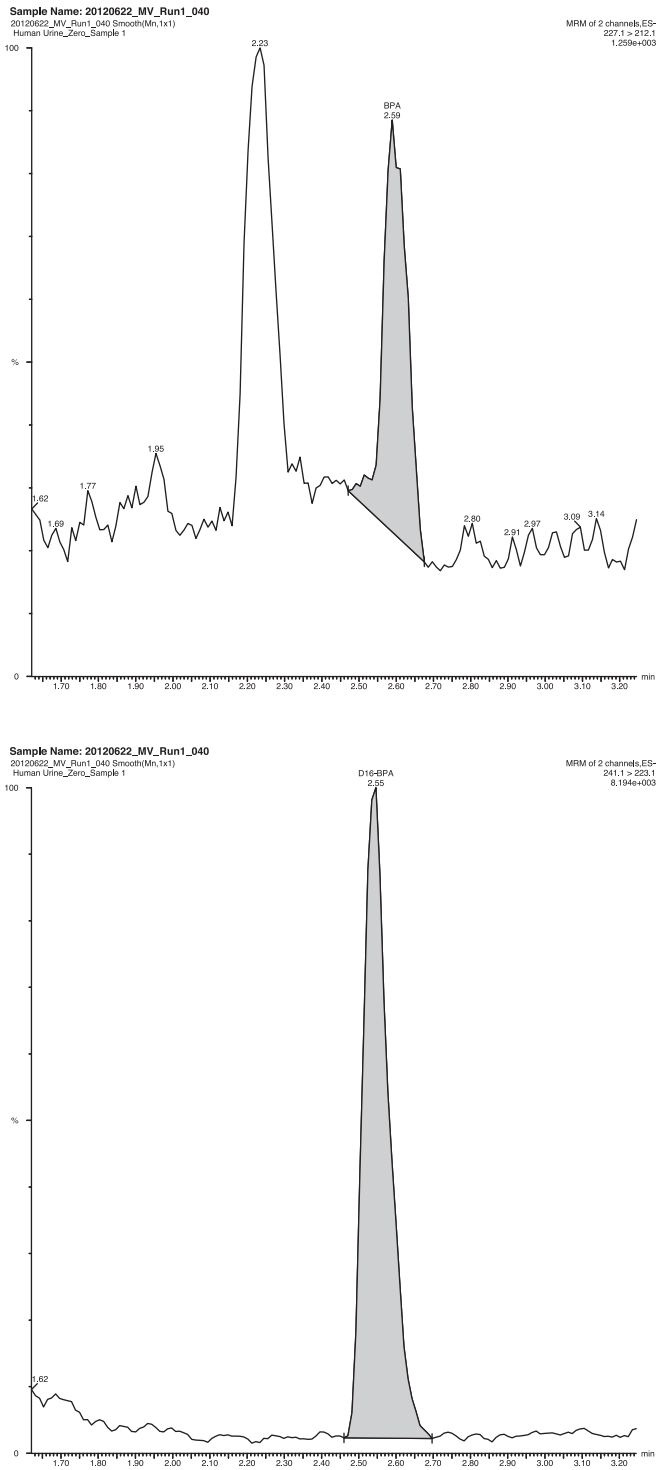

Fig. 4. SRM mass chromatogram of human urine fortified with IS (zero sample).

Analytical QC results for synthetic urine and human urine are shown in Tables 1 and 2, respectively. All of the individual QC results were within ±20% of nominal concentration, except one QC in the second validation run and three in the third validation run (see Table 2). Mean deviations from nominal and coefficients of variation (CV) were acceptable for all three validation runs. There was a preparation error in the third validation run that impacted two synthetic urine QCs. The BPA-g check samples averaged 96.9% conversion with a CV of 6.02% across the three core validation runs plus the three long-term matrix stability runs.

Integrity of dilution was acceptable with mean deviations from nominal ($N = 6$) of −5.91% and −15.8% for synthetic urine and human urine 50 ng/mL QCs, respectively. Selectivity was acceptable for all six lots of human urine fortified at 2.25 ng/mL. The results ranged from 82.0% to 101.5% of the adjusted nominal concentration.

Re-injection reproducibility met acceptance criteria (two-thirds overall and one QC at each concentration level within ±20% of nominal concentration). Both the synthetic urine and human urine QCs quantitated lower upon re-injection, and this was attributable to the difference in slopes of the calibration curves between the

Table 1
Method validation synthetic urine QC results.

	BPA concentration (ng/mL)		
	QC-L	QC-M	QC-H
Nominal conc.	2.25	8	16
Validation Run 1	2.02	7.21	14.4
	2.22	7.64	14.9
	2.17	7.24	14.6
	2.07	7.30	13.9
	2.09	7.38	13.8
	2.07	7.53	14.7
Mean	2.11	7.38	14.4
CV (%)	3.51	2.31	3.09
Mean accuracy (%)	93.6	92.3	89.9
Validation Run 2	2.21	7.07	13.3
	2.04	7.42	14.2
	1.97	7.51	14.5
	1.87	6.96	15.1
	1.92	6.64	14.6
	1.83	7.34	14.8
Mean	1.97	7.16	14.4
CV (%)	6.97	4.60	4.33
Mean accuracy (%)	87.7	89.5	90.1
Validation Run 3	2.07	PE	16.0
	2.06	7.05	16.6
	2.03	7.13	16.6
	2.34	PE	15.8
	2.19	7.43	17.3
	2.11	9.12	16.1
Mean	2.13	7.68	16.4
CV (%)	5.41	12.7	3.34
Mean accuracy (%)	94.8	96.0	102.5
Overall mean	2.07	7.37	15.1
Overall CV (%)	6.16	7.16	7.28
Overall mean accuracy (%)	92.0	92.2	94.2

CV = (Std Dev/mean) × 100.
Mean accuracy = (mean/nominal) × 100.
PE: prep. error (see text).

Table 2
Method validation human urine QC results.

	BPA Concentration (ng/ml)		
	QC-L	QC-M	QC-H
Nominal Conc. Run 1	2.87	8.62	16.6
Validation Run 1	2.40	6.93	14.5
	2.40	7.10	15.2
	3.06	7.80	14.5
	2.63	7.72	14.2
	2.45	8.21	14.3
	2.76	7.90	15.6
Mean	2.62	7.61	14.7
CV (%)	9.95	6.48	3.78
Mean Accuracy (%)	91.2	88.3	88.5
Nominal Conc. Run 2	2.91	8.66	16.7
Validation Run 2	2.51	7.27	14.0
	2.51	7.71	14.4
	2.39	7.64	13.3[a]
	2.72	7.61	15.2
	2.42	7.86	14.8
	2.43	7.89	14.3
Mean	2.50	7.66	14.5
CV (%)	4.80	2.92	3.21
Mean Accuracy (%)	85.8	88.5	87.3
Nominal Conc. Run 3	3.00	8.75	16.7
Validation Run 3	2.53	6.13[a]	14.8
	2.53	7.58	13.6
	2.47	7.12	15.3
	2.52	7.12	14.2
	2.78	7.69	16.3
	2.27[a]	6.85[a]	15.1
Mean	2.57	7.38	14.9
CV (%)	4.76	4.08	6.27
Mean Accuracy (%)	85.6	84.3	88.9
Overall Nominal	2.93	8.68	16.7
Overall Mean	2.56	7.57	14.7
Overall CV (%)	7.00	4.76	4.52
Overall Mean Accuracy (%)	87.4	87.3	88.3

CV = (Std Dev/Mean) × 100.
Mean accuracy = (mean/nominal) × 100.
[a] >20% deviation.

original injection and the re-injection. The calibration slope for the re-injection was 15% greater. The lower concentrations were not reflective of extract instability, based upon the observed peak areas for the analyte and internal standard which were greater in the re-injection.

The results from the combined recovery and matrix effect experiment showed average values ($N = 6$ at three QC concentrations) in synthetic urine of 89.1% and 91.0% for BPA and d_{16}-BPA, respectively. Average values were 84.1% and 87.5% for BPA and d_{16}-BPA, respectively, in human urine with the BPA result corrected for the measured amount of BPA in the control human urine pool.

Bench-top and freeze/thaw stability in human urine were acceptable with results comparable to the human urine analytical QCs. Mean accuracy ($N = 3$) after 24 h at room temperature was 85.9% of nominal at 2.25 ng/mL and 92.2% of nominal at 16 ng/mL. After three freeze/thaw cycles, the mean accuracy ($N = 3$) was 86.6% and 88.7% at 2.25 and 16 ng/mL, respectively. Long-term matrix stability in human urine QCs was demonstrated at both QC levels (2.25 and 16 ng/mL) with no evidence of instability at either −20 °C or −80 °C (Table 3).

The stored 10 ng/μL BPA working solution (−20 °C for 358 days) quantified 12% higher than a freshly-prepared working solution from a fresh stock. This result may be reflective of differences in the preparation of the two stock solutions, or an increase in concentration of the older working solution from solvent evaporation during usage. In either case, there was no evidence of BPA working solution instability. The BPA-g working solution stability experiment showed no evidence of room temperature hydrolysis of BPA-g to BPA in solution after 20 h. The treatments without enzyme showed only minor BPA responses which were comparable to synthetic urine zero samples. The treatments with enzyme showed 98.9% and 103% conversion for the −20 °C solution and the ambient temperature solution, respectively.

3.2. Application to human urine samples

Following validation, the method was successfully applied to a long-term biomonitoring study. Currently, over 1750 human urine samples have been analyzed in 40 analytical runs. This has been accomplished with a single analytical column, and routine replacement of the guard column has been a key component in maintaining analytical column performance. The guard column is changed when chromatographic peaks begin to show evidence of tailing.

Conversion of BPA-g to BPA has averaged 98.0% with a CV of 6.80% across the 40 runs. The median urine BPA concentration was 2.71 ng/mL. Sample concentrations averaged 4.75 ng/mL with 11% of the samples quantifying below the LLOQ and 2.5% of the samples quantifying above the upper limit of quantitation (ULOQ). Samples quantifying greater than 20% above the ULOQ (1.5% of the total) were re-assayed with appropriate dilution in synthetic urine.

Table 3
Method validation human urine QC LTMS results.

	BPA concentration (ng/mL)			
	QC-L (−20)	QC-H (−20)	QC-L (−80)	QC-H (−80)
QC nominal conc. 30 days	2.78	16.5	2.78	16.5
Validation Run 4[a]	2.49	14.6	2.72	15.4
	2.38	15.3	2.61	15.8
	2.51	15.8	2.63	15.6
	2.53	15.2		
	2.68	14.7		
	2.69	15.7		
Mean	2.55	15.2	2.65	15.6
CV (%)	4.68	3.26	2.21	1.28
Mean accuracy (%)	91.6	92.1	95.5	94.4
QC nominal conc. 92 days	2.76	16.5	2.76	16.5
Validation Run 5	2.59	15.3	2.92	16.7
	2.49	15.1	2.73	15.3
	2.55	14.1	2.77	17.0
Mean	2.54	14.8	2.81	16.3
CV (%)	1.98	4.33	3.57	5.56
Mean accuracy (%)	92.0	89.8	102	98.9
QC nominal conc. 218 days	2.82	16.6	2.82	16.6
Validation Run 6	2.69	15.4	2.66	15.6
	2.55	14.9	2.59	15.9
	2.54	15.7	2.65	15.4
Mean	2.59	15.3	2.63	15.6
CV (%)	3.23	2.64	1.44	1.61
Mean accuracy (%)	91.9	92.5	93.3	94.3

CV = (Std Dev/mean) × 100.
Mean accuracy = (mean/nominal) × 100.
[a] $N = 6$ QCs @ −20 as part of recovery experiment.

4. Conclusion

A method for measuring total BPA in human urine using UHPLC–MS/MS after liquid/liquid extraction was developed and validated over the concentration range of 0.75–20 ng/mL. Matrix stability in human urine was demonstrated after 24 h at ambient temperature, after three freeze/thaw cycles, and after frozen storage at −20 °C and −80 °C for up to 218 days. The method is simple (liquid/liquid extraction) and fast (shorter LC–MS/MS run time than previous methods) with comparable sensitivity. It has been successfully applied to a large biomonitoring study.

Acknowledgment

Financial support for this research came from the National Institute of Environmental Health Sciences – National Institutes of Health (grant R01ES020488).

References

[1] I.T. Cousins, C.A. Staples, G.M. Klečka, D. Mackay, Hum. Ecol. Risk Assess. 8 (2002) 1107.
[2] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Reprod. Toxicol. 24 (2007) 139.
[3] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J.R. Paumgartten, G. Schonfelder, Environ. Health Perspect. 118 (2010) 1055.
[4] Toxicological and Health Aspects of Bisphenol A, Report of Joint FAO/WHO Expert Meeting, Ottawa, Canada, 2–5 November, 2010 http://whqlibdoc.who.int/publications/2011/97892141564274_eng.pdf
[5] J.G. Hengstler, H. Foth, T. Gebel, P.J. Kramer, W. Lilienblum, H. Schweinfurth, W. Völkel, K.M. Wollin, U. Gundert-Remy, Crit. Rev. Toxicol. 41 (2011) 263.
[6] K.A. Langdon, M. StJ. Warne, R.J. Smernik, A. Shareef, R.S. Kookana, Sci. Total Environ. 447 (2013) 56.
[7] O. Ballesteros, A. Zafra, A. Navalón, J.L. Vílchez, J. Chromatogr. A 1121 (2006) 154.
[8] G.W. Lien, C.Y. Chen, G.S. Wang, J. Chromatogr. A 1216 (2009) 956.
[9] H.G. Ayala, E. Moyano, M.T. Galceran, J. Chromatogr. A 1217 (2010) 3511.
[10] R.S. Zhao, X. Wang, J.P. Yuan, J. Sep. Sci. 33 (2010) 1652.
[11] W. Völkel, N. Bittner, W. Dekant, Drug Metab. Dispos. 33 (2005) 1748.
[12] X. Ye, Z. Kuklenyik, L.L. Needham, A.M. Calafat, Anal. Chem. 77 (2005) 5407.
[13] X. Ye, Z. Kuklenyik, L.L. Needham, A.M. Calafat, Anal. Bioanal. Chem. 383 (2005) 638.
[14] W. Völkel, M. Kiranoglu, H. Fromme, Toxicol. Lett. 179 (2008) 155.
[15] X. Ye, L.J. Tao, L.L. Needham, A.M. Calafat, Talanta 76 (2008) 865.
[16] Y. He, M. Miao, L.J. Herrinton, C. Wu, W. Yuan, Z. Zhou, D.K. Li, Environ. Res. 109 (2009) 629.
[17] H. Chang, Y. Wan, J. Naile, X. Zhanga, S. Wiseman, M. Hecker, M.H.W. Lam, J.P. Giesy, P.D. Jones, J. Chromatogr. A 1217 (2010) 506.
[18] D.A. Markham, J.M. Waechter Jr., M. Wimber, N. Rao, P. Connolly, J.C. Chuang, S. Hentges, R.N. Shiotsuka, S. Dimond, A.H. Chappelle, J. Anal. Toxicol. 34 (2010) 293.
[19] X. Jing, S. Bing, W. XiaoYan, S. XiaoJie, W. YongNing, Biomed. Environ. Sci. 24 (2011) 40.
[20] M.Z. Lacroix, S. Puela, S.H. Collet, T. Corbela, N. Picard-Hagen, P.L. Toutain, C. Viguiéa, V. Gayrardb, Talanta 85 (2011) 2053.
[21] J.G. Teeguarden, A.M. Calafat, X. Ye, D.R. Doerge, M.I. Churchwell, R. Gunawan, M.K. Graham, Toxicol. Sci. 123 (2011) 48.
[22] W. Völkel, M. Kiranoglu, H. Fromme, Environ. Res. 111 (2011) 143.
[23] S.H. Yang, A.A. Morgan, H.P. Nguyen, H. Moore, B.J. Figard, K.A. Schug, Environ. Toxicol. Chem. 30 (2011) 1243.
[24] X. Ye, L.Y. Wong, A.M. Bishop, A.M. Calafat, Environ. Health Perspect. 119 (2011) 983.
[25] Z. Zhang, H. Alomirah, H.S. Cho, Y.F. Li, C. Liao, T.B. Minh, M.A. Mohd, H. Nakata, N. Ren, K. Kannan, Environ. Sci. Technol. 45 (2011) 7044.
[26] M. Chen, L. Tao, E.M. Collins, C. Austin, C. Lu, J. Chromatogr. B 904 (2012) 73.
[27] M. Chen, P. Zhu, B. Xu, R. Zhao, S. Qiao, X. Chen, R. Tang, D. Wu, L. Song, S. Wang, Y. Xia, X. Wang, J. Anal. Toxicol. 36 (2012) 608.
[28] I. Kosarac, C. Kubwabo, K. Lalonde, W. Foster, J. Chromatogr. B 898 (2012) 90.
[29] C. Liao, K. Kannan, Environ. Sci. Technol. 46 (2012) 5003.
[30] T. Geens, L. Goeyens, K. Kannan, H. Neels, A. Covaci, Sci. Total Environ. 435/436 (2012) 30.
[31] Y.J. Lee, H.Y. Ryu, H.K. Kim, C.S. Min, J.H. Lee, E. Kim, B.H. Nam, J.H. Park, J.Y. Jung, D.D. Jang, E.Y. Park, K.H. Lee, J.Y. Ma, H.S. Won, M.W. Im, J.H. Leem, Y.C. Hong, H.S. Yoon, Reprod. Toxicol. 25 (2008) 413.
[32] J.M. Braun, A.E. Kalkbrenner, A.M. Calafat, K. Yolton, X. Ye, K.N. Dietrich, B.P. Lanphear, Pediatrics 128 (2011) 873.

[33] J.M. Braun, A.E. Kalkbrenner, A.M. Calafat, J.T. Bernert, X. Ye, M.J. Silva, D.B. Barr, S. Sathyanarayana, B.P. Lanphear, Environ. Health Perspect. 119 (2011) 131.
[34] M.K. Morgan, P.A. Jones, A.M. Calafat, X. Ye, C.W. Croghan, J.C. Chuang, N.K. Wilson, M.S. Clifton, Z. Figueroa, L.S. Sheldon, Environ. Sci. Technol. 45 (2011) 5309.
[35] L.N. Vandenberg, I. Chahoud, V. Padmanabhan, F.J.R. Paumgartten, G. Schoenfelder, Environ. Health Perspect. 118 (2010) 1051.
[36] W. Dekant, W. Völkel, Toxicol. Appl. Pharmacol. 228 (2008) 114.
[37] National Research Council, National Academy of Sciences, Human Biomonitoring for Environmental Chemicals, National Academies Press, Washington, DC, 2006.
[38] Chemistry and Analytical Methods for Determination of Bisphenol A in Food and Biological Samples, FAO/WHO Expert Meeting on Bisphenol A (BPA), Ottawa, Canada, 2–5 November, 2010 http://www.who.int/foodsafety/chem/chemicals/1_chemistry_analytical_methods.pdf
[39] J.J. Pritchett, R.K. Kuester, I.G. Sipes, Drug Metab. Dispos. 30 (2002) 1180.
[40] W. Jian, R.W. Edom, N. Weng, Bioanalysis 4 (2012) 2431.
[41] M. Shimizu, K. Ohta, Y. Matsumoto, M. Fukuoka, Y. Ohno, S. Ozawa, Toxicol. In Vitro 16 (2002) 549.
[42] U.S. Department of Health and Human Services, Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, Center for Drug Evaluation and Research (CDER), Rockville, MD, 2001 http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf
[43] Scientific Working Group for Forensic Toxicology (SWGTOX), Standard Practices for Method Validation in Forensic Toxicology, 2013.