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Quantification of deuterated bisphenol A in serum, tissues, and excreta from adult Sprague-Dawley rats using liquid chromatography with tandem mass spectrometry†

Nathan C. Twaddle, Mona I. Churchwell, Michelle Vanlandingham and Daniel R. Doerge*

Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

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Bisphenol A (BPA) is an important industrial chemical used in the manufacture of polycarbonate plastic products, epoxy resin-based food can liners, and paper products. The presence of BPA in urine of >90% of Americans aged 6–60 suggests ubiquitous and frequent exposure and is problematic because of the potential for endocrine disruption. The ubiquity of environmental BPA in common laboratory supplies used for sample collection, storage, and analysis greatly increases the likelihood of false positive determinations, particularly at trace levels. The current study validated using liquid chromatography/tandem mass spectrometry (LC/MS/MS) in conjunction with deuterated BPA as the dosing material to circumvent contamination for high sensitivity quantifications in rat serum, tissues, urine, and feces. The methods described provided measurements of both estrogen receptor-active aglycone and metabolically deactivated conjugated forms of BPA, a distinction that is critical to assessing toxicological potential. The adequacy of the described methodology was substantiated by its utility in analyzing samples from rats treated orally with a 100 μg/kg body weight dose of d6-BPA. These results emphasize the challenges inherent in measuring BPA in biological samples and how employing stable isotope labeled dosing can facilitate pharmacokinetic studies needed to understand BPA metabolism and disposition. Such studies conducted in experimental animal models, in conjunction with properly validated human biomonitoring data, will be the basis for PBPK modeling of BPA in environmentally exposed humans. Published in 2010 by John Wiley & Sons, Ltd.

Bisphenol A (2,2-bis-(*p*-hydroxyphenyl)-2-propane, BPA) is one of the highest production volume industrial chemicals worldwide ($>10^9$ kg/year). BPA is the monomer used for synthesis of polycarbonate plastics and its glycidyl ethers are used for synthesis of epoxy resins.[1] Shatter-proof polycarbonate plastic is used in many common consumer products, including beverage containers and medical devices. Epoxy resins are used as liners for water pipes, metal cans for food and beverages, and as dental sealants. Hydrolysis or leaching of unreacted monomer can release low levels of BPA that lead to human exposure. Of particular concern are products affecting young children, including infant formula,[2] polycarbonate bottles,[3] and medical devices used in neonatal intensive care units (NICU[4]). Various paper products are additional sources of environmental exposure, including thermal paper used for cash register receipts and books.[1,5] In the U.S. population, urinary biomonitoring indicates that current adult exposure to BPA, estimated at ≤1 μg/kg body weight (bw)/day, is frequent and pervasive.[6,7] Children typically have the highest exposures, with daily estimates from all sources in the range of 0.1–10 μg/kg bw/day.[1] These exposures are problematic because a wide body of evidence suggests the possibility of endocrine disruption, particularly during the perinatal period when developmental programming occurs (reviewed in Richter *et al.*[8]).

The analytical basis for the preponderance of BPA biomonitoring is liquid chromatography with tandem mass spectrometric detection (LC/MS/MS), most often using stable isotope labeled internal standards for isotope dilution quantification. The sensitivity and selectivity afforded by selected reaction monitoring (SRM) methods are essential for validated measurements of BPA in biological samples, especially when the data are used for risk assessment. These measurements are critical because they can provide information on the levels of aglycone BPA, which is the estrogen receptor-active species in tissues, and its inactive phase II conjugates (glucuronide and sulfate), which are excreted in urine. The most successful methodology used for BPA biomonitoring involves dedicated on-line sample preparation antecedent to MS/MS with SRM detection.[9] However, the ubiquity of BPA in the environment,

**Correspondence to*: D. R. Doerge, Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA.
E-mail: daniel.doerge@fda.hhs.gov

including analytical laboratories, makes the measurement of low levels of native BPA problematic due to a propensity for false positive determinations, particularly when using highly sensitive detection methods like LC/MS/MS in SRM mode.[10,11] The presence of non-zero responses for BPA and other xenobiotic phenolics from system blanks,[9] even when using extreme caution against the introduction of such contaminants, emphasizes the high potential for contamination by aglycone BPA during all stages of sample collection, storage, and analysis. Strict attention to issues of sample integrity is also emphasized by the high prevalence of BPA in samples collected from indoor and outdoor air, house dust, and surface wipes.[12]

The current study reiterates both the challenges inherent in conducting measurements of BPA in biological samples and how the use of stable isotope labeled dosing can facilitate pharmacokinetic studies. Pharmacokinetic measurements of both aglycone (i.e., active) and conjugated (i.e., inactive) forms of BPA in serum, tissues, and excreta from animal models are critical information needed to understand BPA metabolism and disposition, particularly as it relates to that in humans. This study provides validated LC/MS/MS methodology to make such measurements that takes into account the ubiquity of BPA contamination and its impact on analytical determinations.

EXPERIMENTAL

Reagents

Isotopically labeled BPA was purchased from Cambridge Isotope Labs (Andover, MA, USA), who supplied the $^{13}C_{12}$-BPA (99% isotopic purity and >98% chemical purity), and CDN (Pointe Claire, Quebec), who supplied the d6-BPA (dimethyl-d6; 98.6% isotopic purity and >99.7% chemical purity). Sigma Chemical Co. supplied all the biochemical reagents. All solvents utilized were HPLC grade and Milli-Q water was used throughout.

Characterization of standards

LC-UV (Shimadzu 10AD, Kyoto, Japan, 275 nm) was utilized to ensure equal concentrations of the two labeled BPA species. A Luna analytical column (2.0 × 150 mm, 3 μ particle size; Phenomenex, Torrance, CA, USA) was used with an isocratic mobile phase consisting of 30% aqueous methanol and a flow of 0.15 mL/min. No unlabeled BPA was detected using LC/MS/MS in the internal standard or the d6 dosing material (<0.1%). Because $^{13}C_{12}$-BPA was received as a certified standard solution, its concentration was used as the reference. The d6-BPA was weighed, dissolved into acetonitrile and the concentration was adjusted based on UV response. Subsequent dilutions were made into methanol. Throughout the study, d6-BPA was accurately weighed for use in dosing solutions and the concentration verified each time using the $^{13}C_{12}$-BPA internal standard.

Liquid chromatography

A Waters Acquity UPLC system (Waters, Inc., Milford, MA, USA) was operated at a flow rate of 0.2 mL/min to deliver a gradient mobile phase beginning at 40% (v/v) aqueous methanol. To maximize the separation of BPA from suppression by unretained interferences, a shallow gradient was introduced at 0.5 min increasing the methanol linearly to 70% (v/v) over the following 9.5 min. Afterwards, a step gradient increased the methanol to 98% (v/v), held for 3 min with a subsequent column re-equilibration to initial conditions. Total run time was 15 min. This mobile phase adequately separated the analyte peak from areas of suppression using a Luna C18(2) analytical column (2.0 × 150 mm, 3 μ particle size; Phenomenex, Torrance, CA, USA) with a SecurityGuard pre-column filter (Phenomenex, Torrance, CA, USA) maintained at 50°C. Injection volumes were 35–50 μL.

Mass spectrometry

A Quattro Premier XE tandem mass spectrometer (Waters Inc., Milford, MA, USA) equipped with an electrospray probe was used in selected reaction monitoring (SRM) mode for analysis of negative ions. Optimal transitions for d6-BPA and $^{13}C_{12}$ -BPA giving the greatest MS response were monitored along with secondary confirmatory transitions (Table 1). Native BPA (molecular weight (mw) 228 Da) was also monitored. Other MS parameters included source and desolvation temperatures of 120°C and 350°C, respectively, argon as collision gas (4.39e-3 mbar), and nitrogen as desolvation gas (800 L/h). A cone potential of 40 V was maintained for all compounds as was a capillary of 3.0 kV. Collision energies of 20 and 25 eV were used for main and confirmatory transitions, respectively. Resolutions were set to give precursor and product ion peak widths of 0.9 Th.

Calibration curve

A calibration curve was prepared by diluting methanolic solutions of BPA into water at the starting mobile phase composition to obtain a MS response ratio over a range of d6-BPA concentrations (a total of 5 calibrators in the range of 0.01–10 ng/mL in duplicate) while keeping the $^{13}C_{12}$-BPA concentration constant (1 ng/mL) and the response ratio was linear over these 3 orders of magnitude. The slope of the calibration curve was 0.98, which was used for all quantifications and was checked daily using prepared standards analyzed before and during sample sets.

Enzymatic deconjugation

Total serum concentrations of BPA (i.e., aglycone + conjugates) were quantified following incubation with a *H. pomatia* glucuronidase/sulfatase mixture (Sigma Sulfatase H-1, 2

Table 1. Selected reaction monitoring (SRM) transitions acquired, optimal cone voltages, and collision energies used

Compound	SRM transition Precursor > fragment	Cone voltage (V)	Collision energy (eV)
BPA	226.9 > 212.0	40	20
d6-BPA	232.9 > 215.0	40	20
d6-BPA (Confirm)	232.9 > 138.0	40	25
$^{13}C_{12}$-BPA	238.9 > 224.0	40	20
$^{13}C_{12}$-BPA (Confirm)	238.9 > 138.9	40	25

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units/assay). This preparation contains sufficient glucuronidase (≥300 units/mg protein) and sulfatase (≥10 units/mg protein) activity for total conjugate hydrolysis. The time course for deconjugation was determined from 0.25–24 h incubations using a pooled monkey serum sample obtained following oral dosing with d6-BPA, which contained primarily conjugated forms of BPA. Maximal enzymatic hydrolysis was observed after 2 h. Conjugate levels determined after total enzymatic hydrolysis were indistinguishable (t-test) from those derived from acid hydrolysis (2 M HCl at 99°C for 2 h; data not shown). Serum concentrations of aglycone BPA were measured without the enzymatic hydrolysis step and no evidence for hydrolysis during sample preparation was observed.

Animal handling procedures

The details for all experimental procedures involving adult female Sprague-Dawley rats were previously described for the serum pharmacokinetic evaluation of the same animals.[14] Following oral dosing, serum samples were obtained by removal from the lateral tail vein. After the completion of each blood withdrawal, rats were placed in metabolism cages for collection of total urine samples (24 h). Total urine volume was recorded. Feces were also collected for this 24 h period but only a limited subsample was analyzed. Tissues, urine, and feces were also collected from untreated control rats.

Analysis in serum

A supported liquid extraction (SLE) method was developed for the analysis of BPA in serum. Thawed serum was briefly vortex mixed and added to a culture tube containing citrate buffer (25 mM, pH 5.0) such that the volume totaled 400 μL. To this mixture was added 30 μL internal standard (10 pg/μL, 43 nM final concentration) prepared in methanol. The solution was then thoroughly mixed and extracted with and without prior enzymatic deconjugation. For samples receiving enzymatic deconjugation, 100 μL of citrate buffer was replaced with 100 μL of glucuronidase/sulfatase (2 U), which was also prepared in citrate buffer and the samples were incubated for 2 h at 37°C. Aliquots of each sample were analyzed for aglycone (≤100 μL) and total (≥10 μL) d6-BPA.

Analyte extraction was achieved using SLE+ plates (400 mg, Biotage, Charlotte, NC, USA) and a 96-well plate vacuum manifold. The buffered solution was added to the sorbent bed with a brief pulse of vacuum to facilitate absorption. After 5 min, 900 μL of methyl *tert*-butyl ether (MTBE) was added and allowed to flow under gravity into a 96-well, 2 mL receiving plate. Once the first aliquot of MTBE had reached the top of the sorbent bed, a second 900 μL of MTBE was added and again allowed to flow under gravity. A brief pulse of vacuum was applied once the second aliquot reached the top of the sorbent bed to ensure the complete flow of solvent through the plate. The resulting eluent solvent tray was transferred to a heated 96-well nitrogen evaporation system (SPEdry 96, Biotage, Charlotte, NC, USA) and was brought to dryness. The plate was allowed to cool and the samples were reconstituted to 100 μL with 40% aqueous methanol, sealed with a cap mat and vortex mixed briefly before being placed into the autosampler for injection. Identical procedures were used to analyze amniotic fluid (not shown).

Analysis in tissue

A portion of tissue (100–200 mg) was accurately weighed into a 2.0 mL polypropylene Eppendorf tube then internal standard was added such that the final concentrations in a total of approximately 330 μL methanol were 33 mg/mL and 30 pg/mg, respectively. Tissues were homogenized using a TissueLyser II (Qiagen, Germantown, MD, USA) with a 5 mm stainless steel ball operated at 30 Hz for 5 min. Following homogenization, the samples were centrifuged for 5 min at 16 600 *g*. A 30 μL aliquot of supernatant was transferred to a glass culture tube containing 470 μL of citrate buffer (25 mM, pH 5.0) and mixed. When performing enzymatic deconjugation, 100 μL of buffer was replaced with glucuronidase/sulfatase (2 U) in citrate buffer and placed into a water bath at 37°C for 2 h. The buffered solution was then processed using solid-phase extraction in 96-well plate format (Discovery SPE, 50 mg, CN; Supelco, Bellefonte, PA, USA). Activation and conditioning consisted of 0.5 mL of 50:50 (v/v) methanol/acetonitrile followed by 2 × 0.5 mL aliquots of water. The sample was then loaded and subsequently washed with 1 × 0.5 mL water followed by 1 × 0.5 mL 20% aqueous methanol. Analyte was eluted using two aliquots of 0.5 mL 50:50 (v/v) methanol/acetonitrile and collected into a 2 mL 96-well sample tray. The eluent was evaporated using a heated nitrogen evaporation system and reconstituted to 100 μL with 40% aqueous methanol. The tray was capped and briefly mixed prior to injection.

Analysis in urine

The CN SPE method described for tissues was also used to quantify BPA in urine. Thawed urine was vortex mixed and an aliquot (10–100 μL) was transferred to a glass culture tube containing citrate buffer (25 mM, pH 5.0) for a total volume of 500 μL. The solution was briefly mixed and extracted after enzymatic deconjugation as described for the tissues.

Analysis in feces

Feces evaluation posed a challenge because of high signal suppression observed in both SLE+ and CN SPE methods. Because of the relatively few samples to be analyzed, a manual liquid-liquid extraction (LLE) method was developed and validated. Portions of numerous feces pellets were combined to obtain a more representative sample. Feces (150–300 mg) were weighed into a 2.0 mL centrifuge tube containing methanol and internal standard such that the final concentrations were 0.2 g/mL and 300 pg/mg, respectively, along with a 5 mm stainless steel ball. The tubes were transferred to the TissueLyser II and were operated at 30 Hz for 5 min. Following homogenization, the samples were centrifuged for 5 min at 16 600 *g*. Afterwards, 5 μL of supernatant (1 mg feces equivalent) was added to a separate 1.5 mL centrifuge tube containing 495 μL citrate buffer. For samples requiring enzymatic deconjugation, a 5 μL aliquot of the supernatant was transferred to a culture tube containing 395 μL citrate buffer and processed as described for the tissues. Following incubation, the samples were transferred into 1.5 mL centrifuge tubes for subsequent LLE. MTBE was added (500 μL) to the tube, which was then placed in a Thermomixer (Eppendorf, Hauppauge, NY, USA) at ambient temperature for agitation at 1400 rpm for 5 min. The organic

layer was removed using a glass Pasteur pipette and transferred to a 2 mL 96-well plate. The extraction process was repeated once more and individual organic portions were combined. Once completed, the plate was transferred to a heated 96-well nitrogen evaporation system and brought to dryness. Extracts were reconstituted in 100 μL of 40% aqueous methanol. The plate was capped and briefly mixed prior to injection.

Quality control

With each sample set analyzed, quality control samples were included consisting of buffer and matrix blanks, spikes, and evaluation of a pool of incurred serum from d6-BPA-dosed monkeys either via intravenous (IV) (containing predominately aglycone) or oral administration (containing predominately conjugates). Tissues and excreta samples from untreated rats were always evaluated to ensure no contamination was present. The pool of IV serum was used as a control for those samples not receiving enzymatic deconjugation and the pool of oral serum was used to monitor enzymatic deconjugation. In addition, injections of neat standard were intermittently placed throughout sample sets to monitor responses for unlabeled and labeled analytes.

RESULTS

Contamination by native BPA

Initially, analytical methodology was evaluated in order to make measurements of aglycone and total BPA in samples derived from pharmacokinetic studies in rodents. It was quickly determined that significant contamination by BPA was problematic in typical protocols used for sample preparation and LC/MS/MS analysis. Figure 1 shows the results from a typical SLE protocol in which levels of aglycone BPA (130 pg on-column) in a 100 μL buffer blank quantified at approximately 1.3 ng/mL (left panel). Similarly, the right panel of Fig. 1 shows SRM chromatograms resulting from manual LLE of a 100 μL buffer blank of aglycone BPA produced in polypropylene tubes (35 pg on-column) that quantified at approximately 0.3 ng/mL, well above the limit of detection (LOD) of approximately 0.05 ng/mL. Sources for the contamination were extensively evaluated and included some, but not all, lots of solvents and plastic sample vials. However, these sources of contamination were generally small compared to that from the sample preparation materials (cf. Figs. 1(A) and 1(B)). Because dosing with stable isotope labeled d16-BPA was previously used successfully in an analysis of pharmacokinetics in humans,[13] this strategy was used in analysis of serum, tissues, and excreta derived from pharmacokinetic studies of rodents[14] and non-human primates[15] using a commercially available d6-BPA. It should be noted that the SRM chromatogram for m/z 233 → 139 in the reagent blank samples shown in Figs. 1(A) and 1(B) (see middle chromatograms) show no responses for d6-BPA.

Method development

Rat serum

The method for analysis of d6-BPA was optimized to maximize overall analyte response while minimizing run time and matrix suppression. This combination was achieved using SLE, which provided a robust and reproducible method and allowed for high analyte recoveries (>90%) with minimal suppression (<35%). Lower limits of quantification, when evaluating 100 μL of serum and using a 10 signal-to-noise (S/N), were approximately 0.08 ng/mL (0.34 nM) and a lower LOD, 3 S/N, of 0.05 ng/mL (0.2 nM). It was observed in samples containing low concentrations of d6-BPA that the confirmatory trace often showed a higher S/N ratio because of lower noise levels. Contamination by native BPA was consistently observed. Figure 2 shows representative chromatograms for aglycone and total d6-BPA in serum collected from a rat 8 h after an oral dose of 100 μg/kg bw.

Rat tissues, urine, and feces

Given the additional requirements to extract BPA from rat tissues, urine, and feces, separate methods were designed and validated to provide adequate sample clean-up and minimize matrix suppression. The extraction methods developed and validated allowed for additional sample-washing steps to further remove suppressing matrix effects as compared to the method used for serum, albeit with a slightly more labor-intensive preparation. Method recoveries for urine, using 100 μL and when compared to a fortified sample, were greater than 95%; however, using this volume produced greater suppression than from an equal volume of serum (approximately 60%). Suppression was minimized when analyzing smaller sample sizes (e.g. 10 μL). Nearly complete suppression was observed when urine was analyzed using the SLE+ plate.

The TissueLyser II provided effective homogenization and allowed for multiple individual samples to be processed simultaneously. The rapid reciprocation of the stainless steel ball efficiently disrupted the tissue, provided for a well-controlled sample preparation in a closed environment, and minimized the potential for cross-contamination. Tissue equivalents of 10 mg were evaluated for liver, brain, and muscle while 1 mg equivalent was evaluated for feces based on pilot evaluations of incurred samples. Methanol was chosen as the homogenization solvent after evaluating various solvents including aqueous buffer and 50% buffered aqueous methanol. The time course for enzymatic hydrolysis was evaluated separately in tissues, feces and urine, using blank samples spiked with small volumes of serum containing known levels of aglycone and total BPA.

Method recoveries for tissues were calculated by comparing a spiked control sample before extraction to a fortified control sample prior to injection. Recoveries of spiked BPA from muscle, brain, and liver were $47 \pm 2\%$, $60 \pm 4\%$, and $99 \pm 4\%$ ($n = 5$), respectively. Matrix suppression was determined to be absent in muscle tissue extracts while brain and liver extracts showed suppression of 7% and 50%, respectively. Liquid-liquid extraction of BPA from feces samples produced recoveries of $87 \pm 3\%$ with matrix suppression of 33%. Lower limits of quantification from analyzing 100 μL of urine, and using a 10 S/N, were approximately 0.45 ng/mL and a lower limit of detection, 3 S/N, of 0.15 ng/mL. Lower limits of quantification from analyzing 1 mg portions of feces were approximately 16 ng/g and a lower limit of detection of 5 ng/g. Brain, liver, and muscle (10 mg portions) gave comparable overall limits of quantification (0.22 ng/g) and detection (0.1 ng/g).

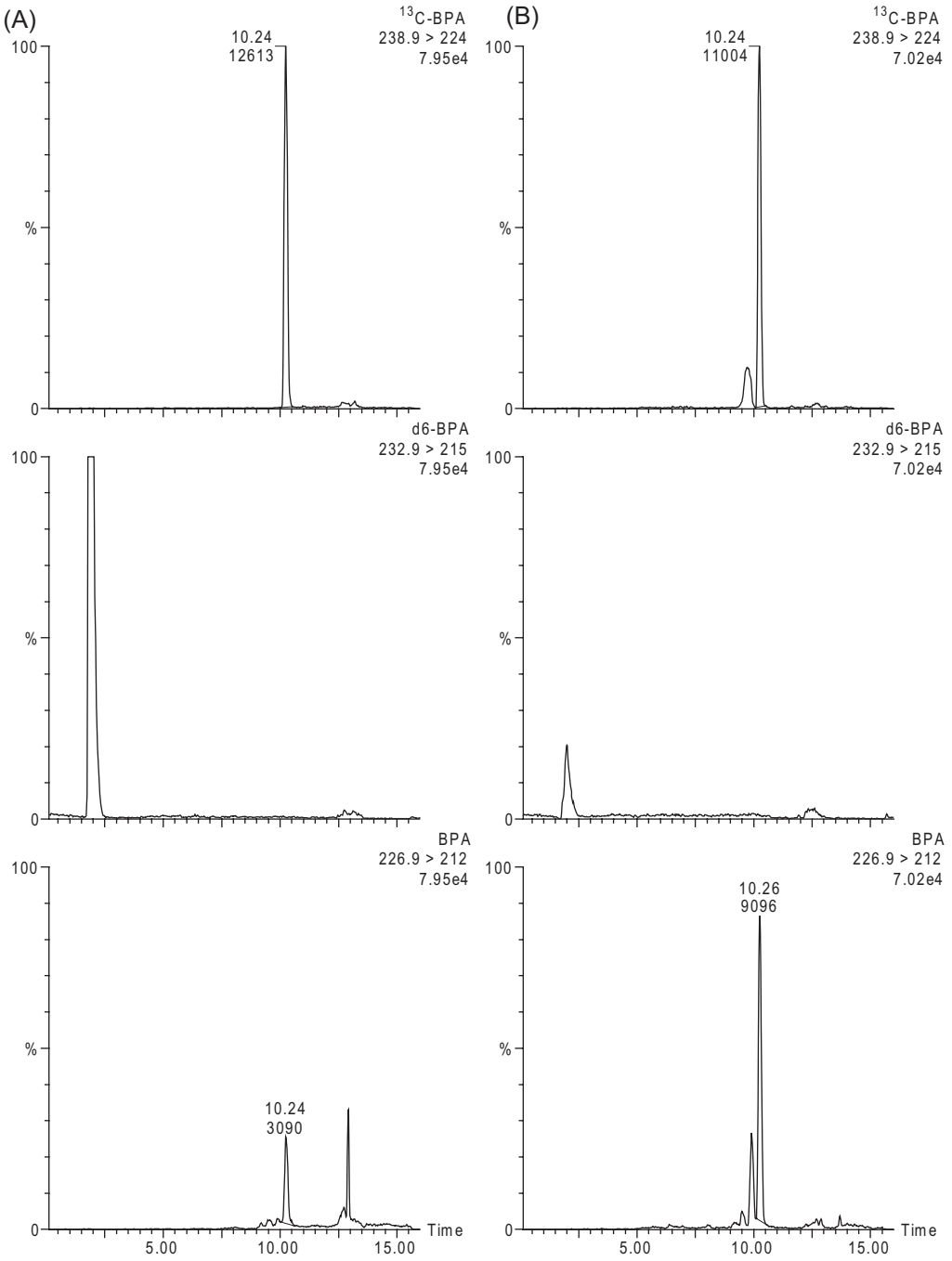

Figure 1. Contamination by native bisphenol A (BPA) in reagent blanks. (A) The bottom trace shows the SRM transition for native BPA contamination (*m/z* 227 → 212) in a blank extraction (100 μL buffer) using MTBE from a polypropylene centrifuge tube corresponding to approximately 0.35 ng/mL (35 pg total). (B) The bottom trace shows the SRM transition for native BPA (*m/z* 227 → 212) in a blank extraction (100 μL buffer) using MTBE through a commercial 96-well supported liquid extraction plate corresponding to approximately 1.3 ng/mL (130 pg total). The traces for *m/z* 239 → 224 and 233 → 215 correspond to $^{13}C_{12}$-BPA (internal standard) and d6-BPA (dosing form). Note: each trace is scaled to its internal standard intensity.

Method validation

Serum

Method validation consisted of spiking replicate control rat serum samples with varying BPA concentrations in quadruplicate over two separate days. Aliquots of 100 μL were processed in quadruplicate and spiked with 0.1, 1.0, and 10.0 ng/mL (0.43–43 nM) d6-BPA with internal standard (IS) at 1 ng/mL (4.3 nM). The results are shown in Table 2. Serum pharmacokinetics derived from such measurements of aglycone and total d6-BPA in rats were previously reported.[14]

Urine

Method validation in urine consisted of spiking control rat urine (100 μL) with varying concentrations, analyzing in

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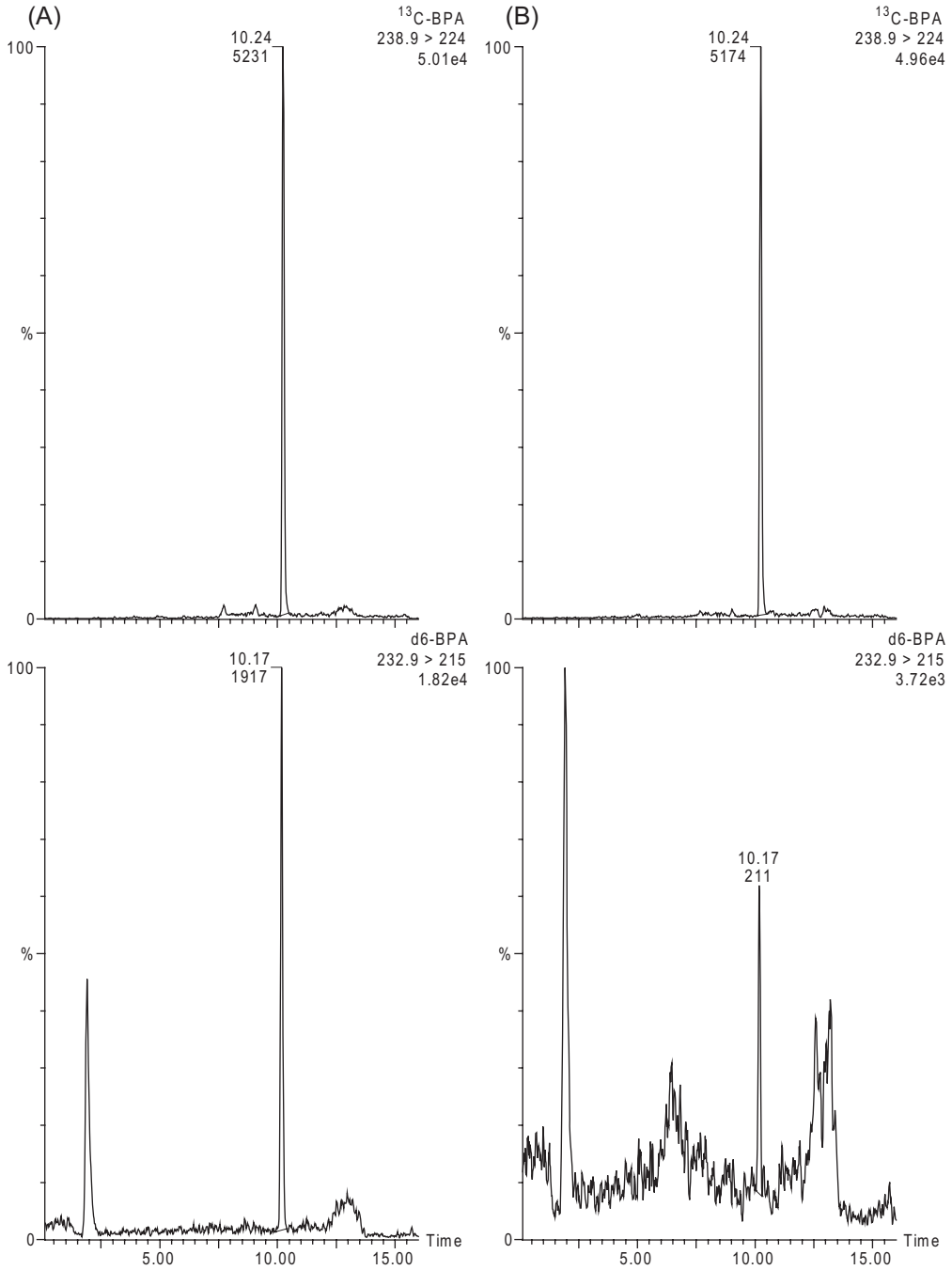

Figure 2. Quantification of total and aglycone bisphenol A (BPA) in serum from a female rat 8 h after oral dosing with d6-BPA (100 μg/kg bw). The left panel (A) shows the analysis of total d6-BPA (45 nM) measured in 10 μL rat serum after total enzymatic hydrolysis using a mixture of glucuronidase and sulfatase enzymes. The right panel (B) shows the corresponding measurement of aglycone d6-BPA (0.5 nM) in 100 μL rat serum without enzymatic hydrolysis treatment.

quadruplicate and repeating the analysis on two separate days. The urine was spiked at 1.0 and 10.0 pg/μL (4.3–43 nM) d6-BPA with an IS at 1 pg/μL (4.3 nM). The results are shown in Table 3. Total d6-BPA was determined in urine collected from the same adult female Sprague-Dawley rats used previously to measure serum pharmacokinetics.[14] Urine collected over 24 h post-dosing contained 329 ± 246 μg/L total d6-BPA (mean ± standard deviation (SD), n = 6 rats), of which 97% was present in conjugated forms.

Tissue and feces

Single concentration validations were conducted for tissues and feces. For tissues, validation consisted of spiking into accurately weighed control tissues 30 pg/mg (130 fmol/mg) IS and 10 pg/mg d6-BPA (43 fmol/mg), homogenizing with the TissueLyser II and processing a 10 mg tissue equivalent through the SPE method. These are shown in Table 4.

Fecal matter was validated by spiking into accurately weighed control feces at 0.2 g/mL with 300 pg/mg

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Table 2. Method validation details for control serum (100 μL, n = 4) spiked at 3 different concentrations of d-BPA (0.43, 4.3, and 43 nM) and evaluated on different days

	Quantified (nM)	
	Day 1	Day 2
Serum	Mean ± SD	Mean ± SD
Spiked amount (nM)	0.42 ± 0.08	0.38 ± 0.05
	4.5 ± 0.42	3.80 ± 0.31
	41.9 ± 1.31	39.3 ± 1.08

Table 4. Method validation details for control tissues (10 mg, n = 5) spiked at a single concentration (43 fmol/mg) and evaluated on two different days

	Quantified (fmol/mg)	
	Day 1	Day 2
	Mean ± SD	Mean ± SD
Liver	37.7 ± 3.141	43.4 ± 3.852
Brain	40.9 ± 2.658	40.4 ± 1.426
Muscle	37.07 ± 1.668	42.5 ± 1.278

(1.3 pmol/mg) of both d6-BPA and IS, homogenizing with the TissueLyser II, and performing LLE on 1 mg feces equivalent. The results are shown in Table 5. Total and aglycone d6-BPA were determined in feces collected from the same adult female Sprague-Dawley rats used previously to measure serum pharmacokinetics.[14] Feces were collected over 24 h and analysis of selected subset samples contained 1.1 ± 0.42 μg/g total d6-BPA (mean ± SD, n = 3 rats), of which 98% was present as the aglycone.

DISCUSSION

As shown in Fig. 1, quantifiable background levels of native BPA are present following LLE with MTBE of a blank sample from polypropylene tubes. Furthermore, even higher levels of contamination were observed in blank samples processed by SLE in standard 96-well plates. These and other preliminary experiments suggested the futility of measuring trace levels of native BPA using typical procedures. For this reason, analytical methodology was developed and validated using d6-BPA. While different techniques were required to achieve the optimal performance (SLE for serum, SPE for urine and tissues, and LLE for feces), acceptable validation results were achieved (see Tables 2–5) to proceed with pharmacokinetic measurements. At the present, significant portions of the work in rodents[14] and non-human primates[15] have been completed. For example, Fig. 2 shows the analysis of aglycone (0.5 nM) and total (45 nM) BPA in serum, collected in an adult female Sprague-Dawley rat 8 h after an oral dose of 100 μg/kg bw d6-BPA, where the conjugated forms are present at ~99%. Similarly, Fig. 3 shows the respective aglycone and total BPA chromatograms for rat urine that contained 92% as conjugates and Fig. 4 shows the respective chromatograms for rat feces that contained 95% as aglycone. The presence of primarily conjugated BPA in urine is a consequence of its enhanced excretion following phase II metabolism in rodents and primates;[13,16,17] however, the aglycone predominates in rat feces because of the effect of enterohepatic recirculation whereby conjugated BPA is secreted in the bile back into the gut where the β-D-glucuronosyl moiety is cleaved by the microflora (reviewed in Willhite *et al.*[17]).

The choice of isotopically labeled d6-BPA as the dosing material was critical for the conduct of these pharmacokinetic studies. Clearly, the ability to circumvent demonstrable levels of background contamination by native BPA was critical to making uncompromised measurements at the lowest possible levels in serum, tissues, and excreta. The sensitivity and specificity of LC/MS/MS using SRM detection made it possible to use an administered dose (100 μg/kg bw) as close as practicable to the range of proposed human exposures while permitting the necessary measurements of both aglycone and conjugated forms of BPA for internal exposure assessments following oral and injection routes of administration.

This report of the adverse influence of pervasive BPA contamination from common laboratory materials once again highlights the high degree of caution required when performing measurements of native BPA, particularly when using highly sensitive LC/MS/MS techniques.[10,13,18] Artifact generation is particularly problematic when measurements of aglycone BPA, which is the contaminating form, are reported without the complementary measurements of the conjugated forms, which predominate in blood and urine. Ye *et al.*[10] reported the analysis of aglycone and total BPA in 15 human serum samples from

Table 3. Method validation details for control urine (100 μL, n = 4) spiked at two different concentrations (4.3 and 43 nM) and evaluated on different days

	Quantified (nM)	
	Day 1	Day 2
Urine	Mean ± SD	Mean ± SD
Spiked amount (nM)	3.9 ± 0.31	4.0 ± 0.27
	40.0 ± 1.35	37.6 ± 2.31

Table 5. Method validation details for control feces (1 mg, n = 4) spiked at a single concentration (1.3 pmol/mg) and evaluated on two different days

	Quantified (pmol/mg)	
	Day 1	Day 2
	Mean ± SD	Mean ± SD
Feces	1.3 ± 0.051	1.3 ± 0.025

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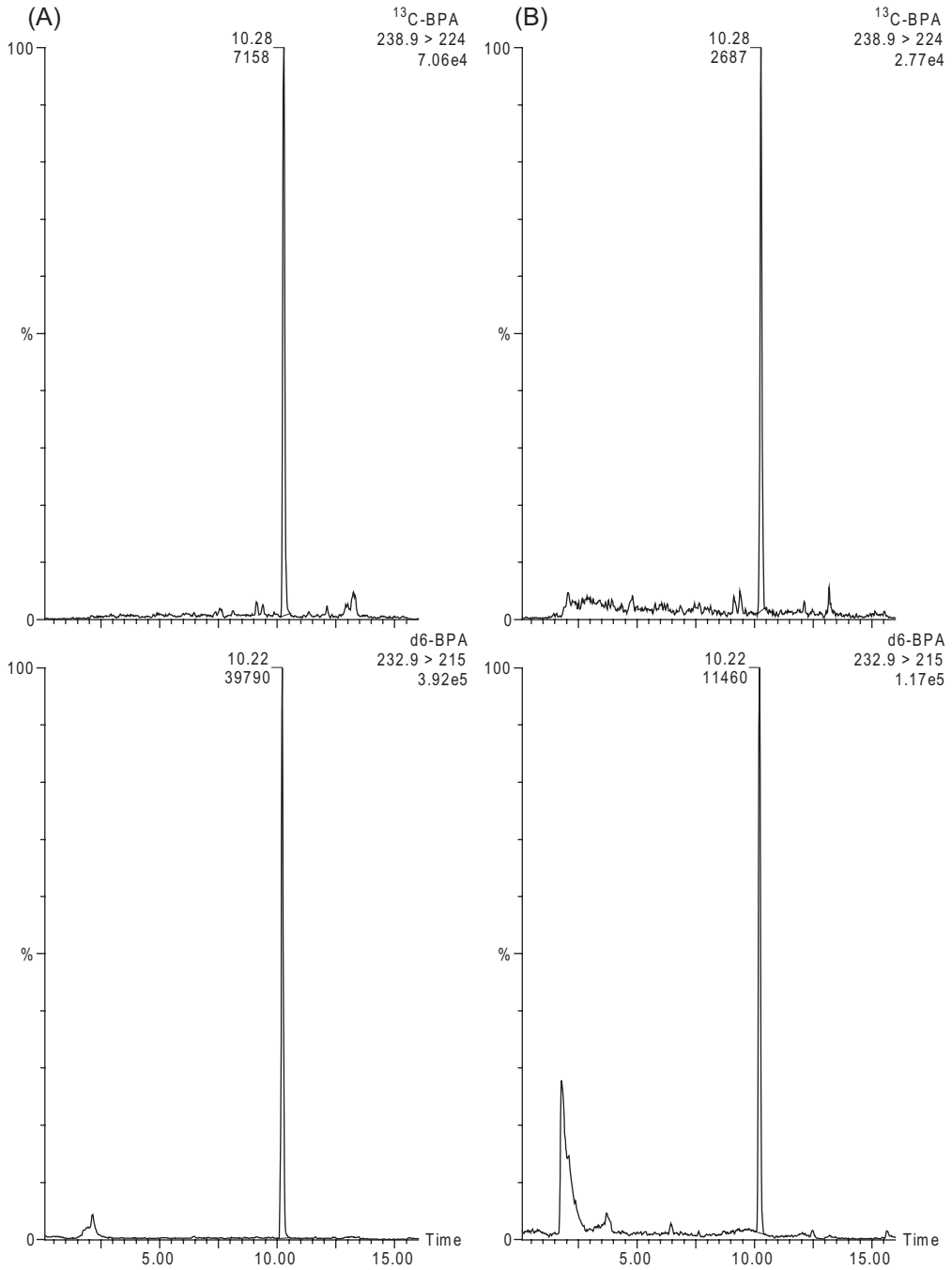

Figure 3. Quantification of total and aglycone bisphenol A (BPA) in 24 h urine sample collected from a female rat after oral dosing with d6-BPA (100 μg/kg bw). The left panel (A) shows the total d6-BPA (694 nM) measured in 10 μL rat urine after total enzymatic hydrolysis using a mixture of glucuronidase and sulfatase enzymes. The right panel (B) shows the corresponding aglycone d6-BPA (54 nM) measured in 100 μL in rat urine without enzymatic hydrolysis treatment.

which only one sample contained measurable BPA. However, in that sample, total and aglycone BPA levels were the same (1.5 ng/mL), an observation made using on-line sample processing and analysis of urine, which is consistent with artifactual hydrolysis or contamination during sample collection, processing, and/or storage.

A large part of the controversy surrounding the risks from environmental exposure to BPA stems from the high percentage of U.S. adults whose urine contains measurable levels of total BPA (93% above the LOD at 0.4 μg/L) in the absence of specific sources of exposure.[6] The most comprehensive human biomonitoring of BPA exposure has come from a large (>2500) nationally representative group of Americans (>6 years of age) performed by the U.S. Centers for Disease Control and Prevention.[6] Measurements made using on-line LC/MS/MS following enzymatic deconjuga-

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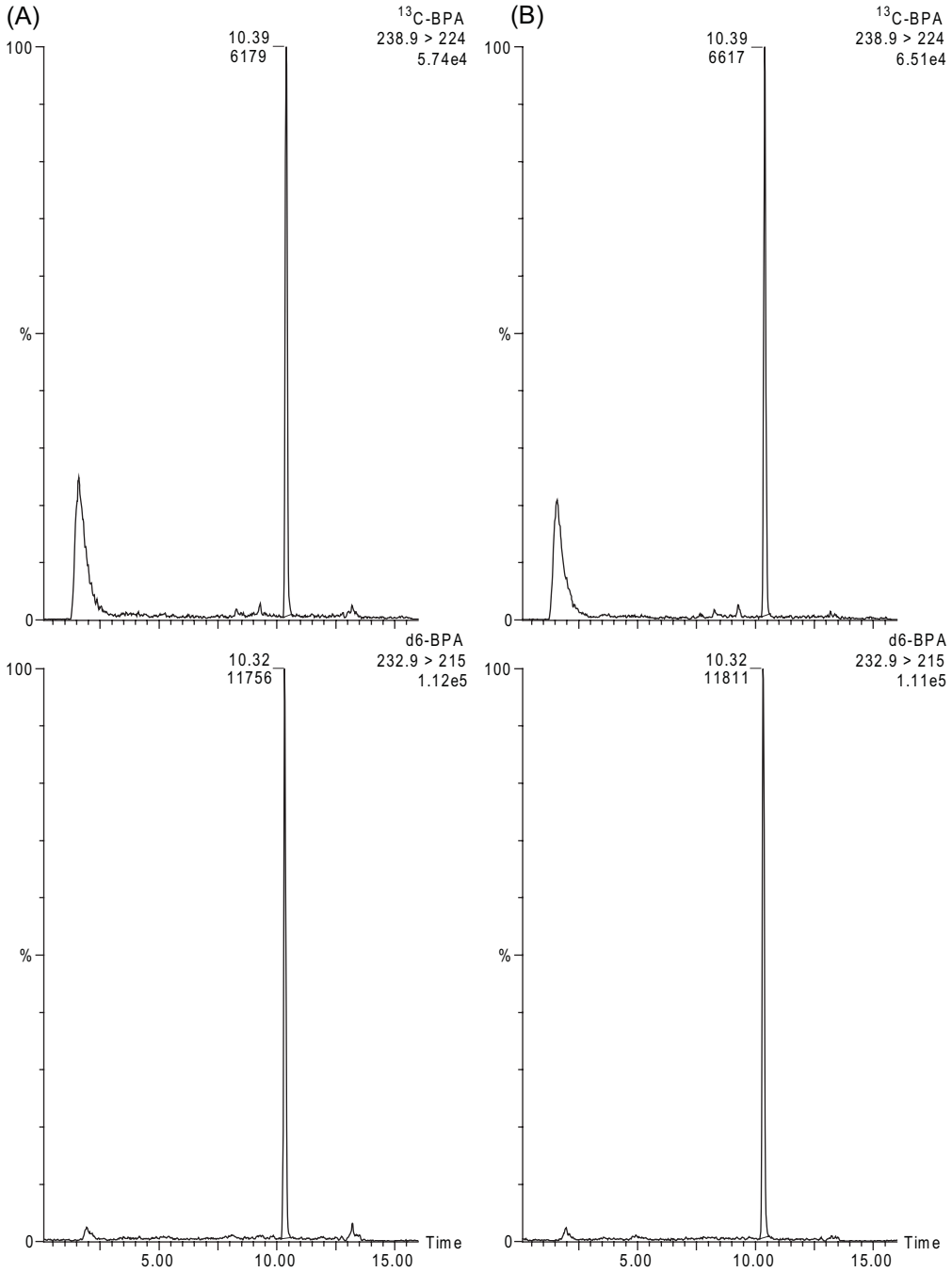

Figure 4. Quantification of total and aglycone bisphenol A (BPA) in 24 h feces sample collected from a female rat after oral dosing with d6-BPA (100 μg/kg bw). The left panel (A) shows the analysis of total d6-BPA (560 nM) measured in 1 mg rat feces after total enzymatic hydrolysis using a mixture of glucuronidase and sulfatase enzymes. The right panel (B) shows the corresponding measurement of aglycone d6-BPA (530 nM) in 1 mg rat feces without enzymatic hydrolysis treatment.

tion of conjugates gave mean concentrations of 2.6 μg/L total urinary BPA. Using these values, in conjunction with estimates of daily urinary output, measured body weights, and the established quantitative extent of excretion as conjugates into human urine,[18] permitted estimation of daily BPA intake in U.S. adults to be less than 0.1 μg/kg bw.[7] A pharmacokinetic modeling analysis of biomonitoring equivalence for BPA suggested that urinary levels of 40 μg/L would result from consumption of 1 μg/kg/bw/day.[19] The 24 h concentrations of total d6-BPA reported here in rats (329 ± 246 μg/L) after a dose of 100 μg/kg bw correspond to approximately 3 μg/L in rat urine per μg/kg bw administered. However, rats excrete only approximately 15% of BPA in urine[20] as opposed to essentially quantitative excretion in humans.[18] Therefore, the adjusted equivalent excretion level in 24 h rat urine assuming 100% urinary

elimination would be approximately 20 μg/L, a value similar to the human biomonitoring equivalent results.[19] This approximation also suggests that a dose of approximately 0.1 μg/kg bw/day would be required to produce the U.S. population mean concentration of 2.6 μg/L total BPA in urine. This intake approximation is consistent with the human BPA intake estimate,[7] although not specifically determined using 24 h urine samples.

Many studies have addressed the presence of BPA in human blood (see reviews by Dekant and Völkel[18] and Vandenberg *et al*.[21]). A number of these studies have reported concentrations of aglycone BPA in human blood in the range of 0.5–3 ng/mL (reviewed in Vandenberg *et al*.[21]). Unfortunately, these studies typically do not report corresponding levels of conjugated BPA making it difficult to assess the potential for hydrolysis or contamination during sample collection, storage, processing, and/or analysis. The critical nature of these methodological constraints is highlighted by the elaborate sample collection, processing, and analytical requirements recently shown to be essential for validated measurements of trace levels of native aglycone BPA in human blood.[22] This distinction is critical because aglycone BPA is the species that binds to estrogen receptors[23] and phase II metabolism to glucuronide and sulfate conjugates is critical in detoxifying BPA and facilitating its excretion. However, under controlled exposure conditions that used stable isotope labeled BPA, other studies have reported undetectable levels of aglycone BPA in human blood (<LOD of 2.3 ng/mL) despite very high levels of conjugated BPA in plasma and urine.[13] These previous findings suggest that blood levels of aglycone BPA in the ng/mL range in environmentally exposed humans would produce far higher urinary levels of conjugated BPA than actually observed.[10] This conclusion is also supported by internal exposure assessments from controlled pharmacokinetic measurements in non-human primates,[15] intravenous infusions in lambs,[24] and biomonitoring equivalence analysis of BPA in humans.[19] The simplest explanation for this discordance is sample contamination leading to false positive determinations of aglycone BPA in human blood, which otherwise would contain levels undetectable by any current analytical methodology.

The LC/MS/MS methods developed and validated here use an isotopic form not susceptible to environmental contamination to provide the basis for ongoing pharmacokinetic measurements in experimental animal models. Such measurements, along with properly validated human biomonitoring data, will be the basis for PBPK modeling of BPA in environmentally exposed humans. In this way, exposures to the active aglycone form of BPA can be estimated in human tissues in order to reduce uncertainty in risk assessments by national and international regulatory bodies.

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