Research Article

Rapid Communications in Mass Spectrometry

Received: 24 August 2015 Revised: 2 November 2015 Accepted: 3 November 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2016**, *30*, 400–406
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7450

Determination of bisphenol A-glucuronide in human urine using ultrahigh-pressure liquid chromatography/tandem mass spectrometry

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RATIONALE: Used widely as a plasticizer and as a monomer for plastics, bisphenol A (BPA) is under investigation as a possible endocrine disrupter. As an indication of systemic exposure, a fast and accurate assay was developed for the major BPA metabolite in human urine, BPA-monoglucuronide (BPA-G), using ultrahigh-pressure liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS).
METHODS: Urine samples were prepared using solid-phase mixed-mode reversed-phase/anion-exchange extraction. BPA-G was measured using UHPLC/MS/MS with an amide UHPLC column interfaced to a triple-quadrupole mass spectrometer equipped with negative ion electrospray, collision-induced dissociation and selected reaction monitoring. [$^{13}C_{12}$]-BPA-G was used as a surrogate standard.
RESULTS: By measuring the glucuronide metabolite of BPA, potential interference due to BPA contamination from containers, solvents, pipette, etc., was avoided. The standard curve had a linear regression coefficient of 0.999, and the intra- and inter-assay variations were less than 10%. The assay was validated according to FDA guidelines.
CONCLUSIONS: A fast, accurate, and highly selective method for the determination of BPA-G in human urine was developed and validated using UHPLC/MS/MS. This method is suitable for assessing human exposure to BPA.

Bisphenol-A (BPA; Fig. 1), an estrogen receptor agonist and endocrine disruptor, is a synthetic plasticizer and monomer used in bottles, medical devices and the linings of beverage and food cans.[1] Because BPA can leach from containers into food or beverages and then be absorbed from the digestive tract after consumption,[2] the main source of BPA exposure has become ingestion from these food and beverage sources.[3] BPA is also added to thermal paper used for cash register receipts, and dermal contact can lead to absorption of BPA through the skin. Studies in Germany[2] and the United States have shown that over 90% of these populations are exposed to BPA, and BPA has been reported to be in the urine of >80% of the population in developed countries.[2,4,5]

BPA has been implicated in contributing to reproductive disorders,[5,15] diabetes,[6,7] cardiovascular disease,[8] and obesity.[9,10] For example, Lang *et al*.[9] reported that Body Mass Index (BMI) is positively correlated with urinary BPA. Similarly, Ning *et al*.[11] showed a positive correlation between the incidence of type 2 diabetes and total BPA in urine. Exposure of children to BPA has also been associated with increased risks of anxiety, depression and hyperactive disorder.[12] To support on-going and future studies of the effects of BPA exposure on human health, accurate measurements of BPA exposure are required.

After absorption, BPA is metabolized in the liver primarily through phase II conjugation to its more polar monoglucuronide (BPA-G), although minor routes of metabolism have been reported including sulfation and hydroxylation (less than 1%).[1,13,14] Small amounts of BPA-G might be deconjugated in the body by β-glucuronidase to reform the aglycon,[1] but BPA-G is the primary hepatic metabolite and is eliminated rapidly in the urine.[15] Because of the ubiquitous use of BPA in plastics found both in the laboratory and in the clinic, contamination of blood and urine specimens acquired for BPA analysis is difficult to prevent. Consequently, human exposure to BPA continues to be uncertain and controversial.[16,17] As a human metabolite not found in laboratory or consumer products, urinary BPA-G is an ideal analyte for the assessment of human exposure to BPA.[3]

A radioactive immunoassay has been developed for the measurement of conjugated BPA, but this method depends upon removal of free BPA from urine prior to measurement and risks overestimation due to laboratory contamination with free BPA.[3] Among the mass spectrometry (MS)-based methods for measuring BPA in urine, those that hydrolyze BPA-G before analysis and then only measure free BPA[18,19] can also overestimate BPA exposure, if environmental BPA leaches into the urine during collection or into the sample

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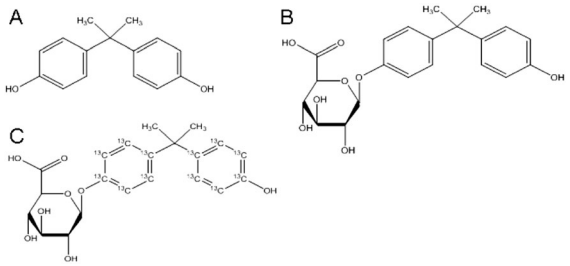

Figure 1. Chemical structures of (A) BPA ($C_{15}H_{16}O_2$; average mass 228.29 g/mol; monoisotopic mass 228.1150 g/mol), (B) BPA-glucuronide ($C_{21}H_{24}O_8$; average mass 404.41 g/mol; monoisotopic mass 404.1471 g/mol), and (C) the surrogate standard [$^{13}C_{12}$]-BPA-G ([$^{13}C]_{12}C_9H_{24}O_8$; average mass 416.32 g/mol; exact mass 416.3223 g/mol).

during laboratory processing. MS-based methods measuring BPA-G directly have used high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) following either acetonitrile dilution[20] or solid-phase extraction (SPE).[1] Our method, validated according to FDA guidelines,[21] uses mixed-mode reversed-phase/anion-exchange SPE to remove potential interfering compounds followed by ultrahigh-pressure liquid chromatography (UHPLC)/MS/MS for faster and higher resolution separation and accurate quantitative analysis of BPA-G.

EXPERIMENTAL

Materials and reagents

BPA-free Honeywell methanol, methyl *tert*-butyl ether, water, and acetonitrile were purchased from VWR International (Radnor, PA, USA). Oasis MAX 3 cc mixed-mode reversed-phase/strong-anion exchange SPE cartridges (60 mg sorbent, 30 µm particle size) were purchased from Waters (Milford, MA, USA). BPA-G and [$^{13}C_{12}$]-BPA-G (Fig. 1) were purchased from Sigma-Aldrich (Steinheim, Germany). Blank human urine was obtained from healthy human volunteers who tested negative for BPA-glucuronide.

Sample preparation

Urine (1 mL) and [$^{13}C_{12}$]-BPA-G (5 µL of 10 µg/mL in methanol) were mixed with 1 mL aqueous ammonia (558 mM). The extraction cartridge was conditioned with 2 mL methanol followed by 2 mL water, and the urine sample was loaded at a flow rate of ≤10 mL/min. The cartridge was washed with 2 mL of aqueous ammonia (290 mM) and then 2 mL of methanol. After drying for 2 min with a stream of air, the cartridge was eluted with 2% formic acid in methanol at a flow rate of ≤5 mL/min. The eluate was evaporated to dryness using a vacuum centrifuge and then reconstituted in 100 µL methanol/water (50:50, v/v) for analysis by UHPLC/MS/MS.

For comparison, BPA-G was extracted from urine specimens using C_{18} SPE. First, the urine was acidified using 1 mL of 240 mM aqueous HCl. The SPE cartridge was then conditioned using 2 mL of methanol followed by 2 mL of water. The urine sample was loaded at a rate of ≤10 mL/min, and the cartridge was washed with 2 mL of water and then 2 mL of methanol. After being dried for 2 min with a stream of air, the cartridge was eluted with methyl *tert*-butyl ether at a flow rate of ≤5 mL/min. The eluate was evaporated to dryness using a vacuum centrifuge and then reconstituted in 100 µL methanol/water (50:50, v/v) for analysis using UHPLC/MS/MS.

Preparation of standards and quality control (QC) samples

Stock solutions of BPA-G were prepared in methanol at a concentration of 328 µg/mL immediately before each set of analyses. The calibration standards were prepared by adding the appropriate amounts of stock solution to pooled blank human urine at final BPA-G concentrations of 1, 5, 10, 25, 50, 100, 250, and 500 ng/mL. The same amount of [$^{13}C_{12}$]-BPA-G standard (5 µL of 10 µg/mL) was added to 1 mL of each sample prior to extraction. QC samples (low, medium, and high) were prepared at BPA-G concentrations of 5, 50, and 500 ng/mL in pooled blank human urine. These samples were also used for recovery experiments. A matrix effect study was carried out using low, medium and high concentrations of BPA-G prepared in water and run through the extraction protocol. All samples were stored at –80°C prior to analysis.

Chromatography

Separations were carried out using a Nexera UHPLC system (Shimadzu, Kyoto, Japan) equipped with a Waters Acquity UPLC BEH amide column (2.1 mm × 100 mm, 1.7 µm). BPA-G was eluted using an 8-min linear gradient from 5% to 40% acetonitrile containing 1 mM ammonium acetate in water at a flow rate of 0.40 mL/min. The column was regenerated by stepping the acetonitrile content to 97% for 0.5 min followed by re-equilibration at 5% acetonitrile for 5 min before the next injection. The injection volume was 2 µL and the column temperature was 20°C. Data acquisition and integration were carried out using Shimadzu Lab Solutions software.

Tandem mass spectrometry

The UHPLC system was interfaced to a Shimadzu LCMS-8040 triple-quadrupole mass spectrometer equipped with negative ion electrospray and operated at unit resolution. Nitrogen was used for nebulization at a flow rate of 3.0 L/min and as a drying gas at a flow rate of 20 L/min. The ion source capillary and vaporizer temperatures were 250 °C. BPA-G was measured using collision-induced dissociation (CID) with selected reaction monitoring (SRM). Argon was used as the collision gas at a pressure of 230 kPa. The SRM transitions for BPA-G were *m/z* 403 to 113 (quantifier) and *m/z* 403 to 175 (qualifier); and the transitions for the surrogate standard [$^{13}C_{12}$]-BPA-G were *m/z* 415 to 113 and *m/z* 415 to 175 for the quantifier and qualifier, respectively. These SRM transitions correspond to fragmentation of the deprotonated molecules (*m/z* 403 and *m/z* 415 for BPA-G and [$^{13}C_{12}$]-BPA-G, respectively) to form dehydrated glucuronate (*m/z* 175) and the characteristic glucuronic acid product ion of *m/z* 113. The SRM dwell time was 35 ms.

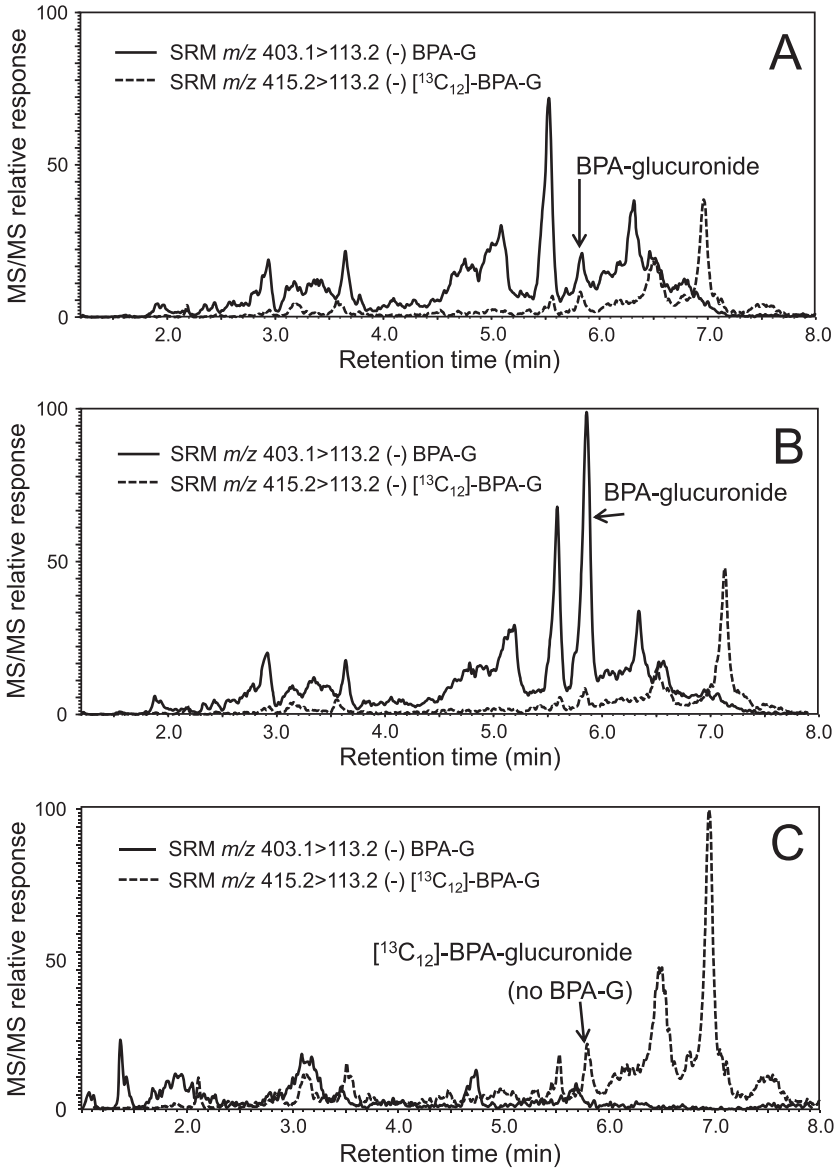

Figure 2. Negative ion electrospray UHPLC/MS/MS SRM chromatograms of BPA-G and [$^{13}C_{12}$]-BPA-G prepared from human urine using mixed-mode reversed-phase/anion-exchange solid-phase extraction: (A) at the LLOQ (5 ng/mL); (B) at a high level of 250 ng/mL BPA-G; and (C) a blank human urine sample containing no detectable BPA-G. Note that UHPLC was effective at separating multiple peaks with similar SRM transitions.

Validation

Linearity, LLOQ, and LLOD

The urine calibration curve was constructed using seven calibration standards in duplicate over four runs. Linearity was determined using least-squares linear regression analysis of the calibration curve. The area ratio of BPA-G to the surrogate standard was plotted against the BPA-G concentration without the use of a weighting factor.

Selectivity

Human urine samples collected from 110 subjects were analyzed for interference at the BPA-G retention time of 5.9 min.

Precision and accuracy

The inter- and intra-assay precision and accuracy were calculated from the standard curve at three concentrations, 5, 100, and 500 ng/mL. The intra-assay precision and accuracy were evaluated for the standard curve by multiple analyses (n = 4) for each point.

Recovery

The recoveries of the mixed-mode reversed-phase/anion-exchange SPE method were determined for three concentrations at the low, middle, and high end of the standard curve. Briefly, blank matrix and spiked matrix were run through SPE, and blank matrix was spiked after SPE. Five

replicates of these recovery experiments were carried out. Recoveries were determined by comparing the UHPLC/MS/MS peak areas of the samples spiked with BPA-G before extraction with those spiked with the same amounts of BPA-G after extraction.

Stability

The freeze/thaw stability of BPA-G was determined over 3 cycles. The long-term stability in urine at –80°C was measured over 28 days. The short-term (24 h) stabilities of BPA-G were determined at room temperature (27°C) and at the autosampler temperature (4°C). The stabilities were determined by comparing the average of all data points (n ≥3) for each non-zero time point with the time zero data point.

RESULTS AND DISCUSSION

Method development

Experiments were carried out to optimize extraction recovery, UHPLC separation and MS/MS sensitivity. BPA-G and [$^{13}C_{12}$]-BPA-G co-eluted under all conditions including the optimized parameters (retention time 5.9 min; Fig. 2). In ~20% of the urine specimens prepared using the C_{18} SPE procedure, a significant interference peak was observed eluting at nearly the same retention time as BPA-G and with similar SRM transitions (Fig. 3). In some samples, this unknown interfering peak was large enough to overlap the BPA-G peak and, if unrecognized, might contribute to an erroneously high measurement of BPA-G. Because attempts to separate BPA-G from this interfering compound using UHPLC alone were unsuccessful, several alternative sample preparation approaches were investigated to remove this compound prior to UHPLC/MS/MS analysis. For example, a previously published acetonitrile dilution approach[20] did not remove this isomeric impurity.

Solid-phase extraction using a mixed-mode reversed-phase/anion-exchange cartridge was, however, found to remove the interfering substance prior to UHPC/MS/MS measurement (Fig. 3). This single extraction approach improved upon a previous method in which two separate extraction treatments were used to remove interfering substances from urine prior to LC/MS/MS analysis.[1] Note that the use of UHPLC instead of HPLC[1,20] was also important for the chromatographic resolution of BPA-G from several other potential interfering substances that still remained in a subset of the urine specimens (Figs. 2 and 3).

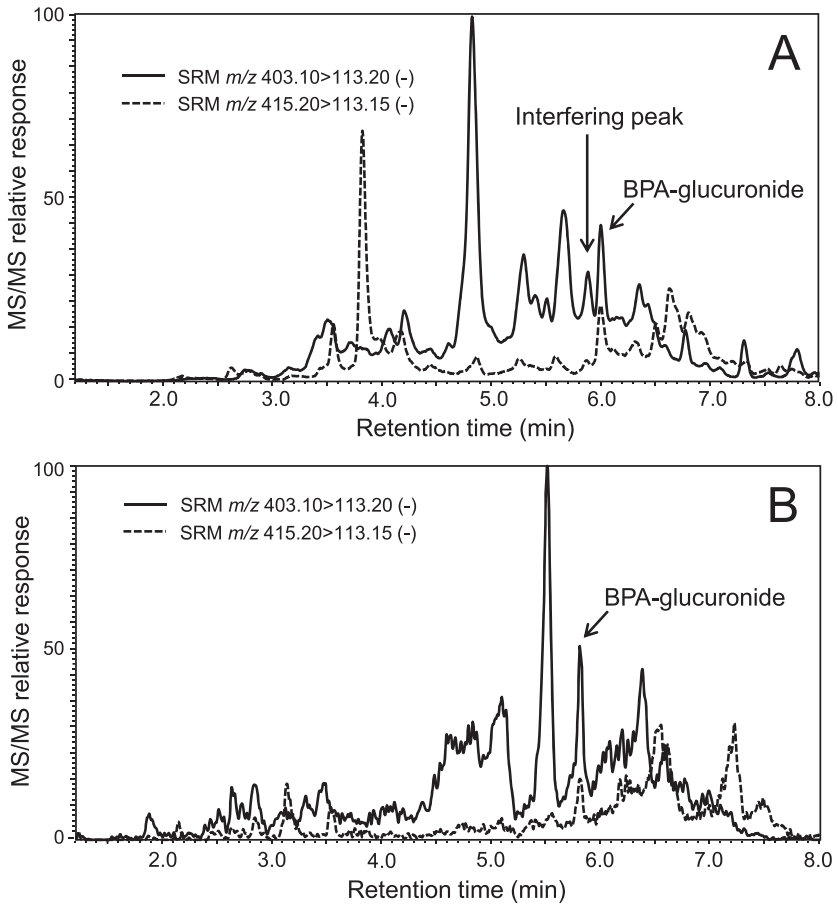

Figure 3. Negative ion electrospray UHPLC/MS/MS SRM chromatograms of human urine extracted using (A) C_{18} solid-phase extraction or (B) mixed-mode reversed-phase/anion-exchange solid-phase extraction. Note the contaminant peak interfering with the measurement of BPA-G that was removed using anion exchange but not reversed-phase solid-phase extraction.

Although greater separation of impurities from BPA-G could be achieved using UHPLC, the conditions were optimized to provide adequate separation with the fastest possible throughput.

Validation

Linearity, LLOQ, and LLOD

The range of the standard curve was from 5 to 500 ng/mL with a linear profile of $r^2 = 0.999$ (Fig. 4). The lower limit of detection (LLOD) was 1 ng/mL, and the lower limit of quantitation (LLOQ) was 5 ng/mL (Fig. 2).

Accuracy and precision

The accuracy of the assay, determined using the percentage difference from nominal values at low, medium, and high levels of BPA-G, ranged from 90.1 to 103.3% (Table 1). The precision was determined using the percentage relative standard deviation and was within 12% (Table 1). Also within FDA guidelines,[21] the inter-assay and intra-assay coefficients of variation (CV) were 2.2% and 5% for 5 ng/mL BPA-G, 3.3% and 9.9% at 100 ng/mL BPA-G, and 0.2% and 7.3% for 500 ng/mL BPA-G, respectively. The assay was applied to the measurement of 110 urine samples that had an average concentration of 58 ng/mL BPA-G, with a range from 6 to 236 ng/mL (39% < LLOD).

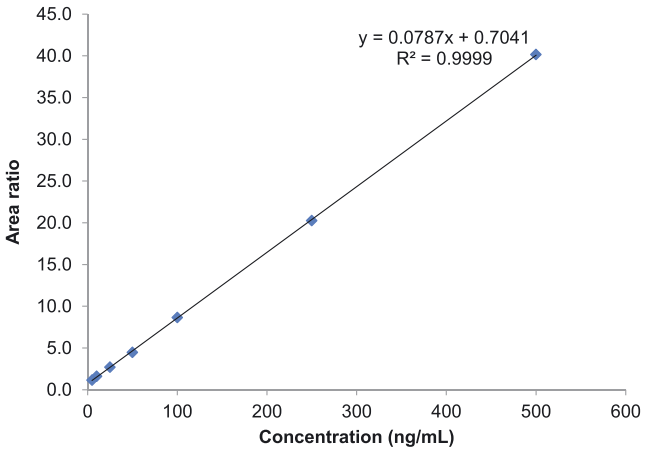

Figure 4. Standard curve for BPA-G (n = 7) obtained using UHPLC/MS/MS with negative ion electrospray showing excellent linearity and sensitivity.

Selectivity

Human urine samples from 110 different individuals were extracted using C_{18} SPE or with a combination of anion-exchange/reversed-phase SPE and evaluated for interferences with an unknown metabolite. An interfering peak eluting at nearly the same retention time as BPA-G was detected in approximately 20% of the urine samples prepared using C_{18} SPE (Fig. 3). Note that this interference might have been missed if only a small set of urine samples had been measured during validation. However, none of the urine samples prepared using anion-exchange/reversed-phase extraction had any observable interference (Figs. 2 and 3).

Recovery

Recovery experiments showed greater than 99% recovery when using anion-exchange/reversed-phase SPE prior to UHPLC/MS/MS analysis (Table 2).

Stability

BPA-G was stable in urine at room temperature for at least 24 h, stable at –80°C for at least 28 days, and stable for at least 3 freeze/thaw cycles (Table 3). Therefore, BPA-G would be stable during the storage and handling conditions required for this method.

Table 1. Intra-assay and inter-assay accuracy and precision for the quantitative analysis of BPA-glucuronide

BPA-glucuronide	Nominal value (ng/mL)	Intra-day (n = 3) CV%	Intra-day (n = 3) Average measured value (ng/mL)	Intra-day (n = 3) Accuracy	Inter-day (n = 6) CV%	Inter-day (n = 6) Average measured value (ng/mL)	Inter-day (n = 6) Accuracy
Low	5	9.1	5.7	95.0	10.7	6.0	102.2
Medium	100	11.2	110.0	90.1	11.7	102.2	103.3
High	500	7.3	500.4	92.7	6.9	503.6	100.2

Table 2. Recovery of BPA-G from human urine at low, medium and high values (ng/mL) after mixed-mode reversed-phase/anion-exchange solid-phase extraction of spiked urine. For comparison, BPA-G was measured in blank urine that was extracted and then spiked with the same amounts of BPA-G standards

BPA-G	Standard (BPA-G added after extraction) (ng/mL) n = 5	Recovered (BPA-G added before extraction) (ng/mL) n = 5	% Recovery
Low	7.85 ± 0.01	7.83 ± 0.01	99.7
Medium	8.69 ± 0.03	9.03 ± 0.03	103.9
High	18.67 ± 0.04	19.69 ± 0.04	104.5

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Table 3. Long- and short-term stabilities of BPA-G in urine (N ≥3)

Initial concentration of BPA-G (ng/mL)	Study	BPA-G remaining ± RSD
5	Freeze-thaw (3 cycles)	98.1% ± 1.9%
	Bench top (27°C, 24 h)	108.8% ± 6.1%
	Long-term (-80°C)	95.2% ± 4.4%
100	Freeze-thaw (3 cycles)	99.3% ± 4.5%
	Bench top (27°C, 24 h)	95.8% ± 3.0%
	Long-term (-80°C, 28 days)	94.9% ± 5.1%
500	Freeze-thaw (3 cycles)	94.6% ± 4.8%
	Bench top (27°C, 24 h)	100.2% ± 0.2%
	Long-term (-80°C, 28 days)	94.0% ± 5.3%

CONCLUSIONS

The urinary metabolite of the plasticizer and endocrine disruptor BPA, BPA-G, may be used as a marker for human exposure to BPA while avoiding contamination problems with sample collection and analysis. This method, validated to FDA standards, utilizes a single-step anion-exchange/reversed-phase solid-phase extraction to remove compounds that were found to interfere even after reversed-phase SPE alone. With a total chromatographic analysis cycle time of less than 14 min, this UHPLC/MS/MS assay is equal to or faster than previous HPLC/MS/MS-based assays for the analysis of BPA-G, while the high chromatographic resolution of UHPLC helps prevent co-elution of other potentially interfering substances. The sensitivity, small sample size (1 mL urine), and fast analysis time make this method attractive for clinical applications for rapid measurement of BPA-G in human urine.

Acknowledgement

The authors would like to thank Shimadzu Scientific for providing the UHPLC/MS/MS system used in this investigation.

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